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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Application Number	10/713,674
Filing Date	November 13, 2003
First Named Inventor	Ken Y. LIN
Group Art Unit	1641
Examiner Name	David J. Venci
Attorney Docket Number	STAN-276

Total Number of Pages in This Submission: **27 pages**

ENCLOSURES (check all that apply)

- [X] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment / Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Documents
- [ ] Response to Missing Parts/ Incomplete Application
  - [ ] Response to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Assignment Papers *(for an Application)*
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s
- [ ] After Allowance Communication to Group
- [ ] Appeal Communication to Board of Appeals and Interferences
- [X] Appeal Communication to Group *(Appeal Notice, Brief, Reply Brief)*
- [ ] Proprietary Information
- [ ] Status Letter
- [X] Other Enclosure(s) *(please identify below):*

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Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Signing Attorney/Agent (Reg. No.)	PAULA A. BORDEN, 42,344 BOZICEVIC, FIELD & FRANCIS, LLP
Signature	
Date	June 1, 2007

EXPRESS MAIL LABEL NO. EV 954 013 599 US

This collection of information is required by 37 CFR 1.5. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2006

Complete if Known	
Application Number	10/713,674
Filing Date	November 13, 2003
First Named Inventor	Ken Y. LIN
Examiner Name	David J. Venci
Art Unit	1641
Attorney Docket No.	STAN-276

☒ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) **250.00**

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	0.00
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims		Extra Claims		Fee ($)		Fee Paid ($)
_____	- 20 or HP =	_____	x	_____	=	0.00

HP = highest number of total claims paid for, if greater than 20

Indep. Claims		Extra Claims		Fee ($)		Fee Paid ($)
_____	- 3 or HP =	_____	x	_____	=	0.00

HP = highest number of independent claims paid for, if greater than 3

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	0.00

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		Extra Sheets		Number of each additional 50 or fraction thereof		Fee ($)		Fee Paid ($)
_____	- 100 =	_____	/ 50 =	_____	(round up to a whole number) x	_____	=	0.00

4. OTHER FEE(S)

	Fee Paid ($)
Non-English Specification, $130 fee (no small entity discount)	_____
Other: **Appeal Brief**	250.00

SUBMITTED BY

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Express Mail No. EV 954 013 599 US

APPELLANTS' BRIEF	Attorney Docket Confirmation No.	STAN-276 9855
	First Named Inventor	Ken Y. Lin
	Application Number	10/713,674
	Filing Date	November 13, 2003
	Group Art Unit	1641
Address to: Mail Stop Appeal Brief-Patents Commissioner for Patents P.O. Box 1450 Alexandria, VA 22313-1450	Examiner Name	D.J. Venci
	Title	*Methods for detecting asymmetric dimethylarginine in a biological sample*

Sir:

This Brief is filed in support of Appellants' appeal from the Examiner's Rejection dated October 18, 2006. No claims have been allowed, and claims 1-9, 15, and 17-19 are pending. Claims 1-9, 15, and 17-19 are appealed. A Notice of Appeal was filed on April 17, 2007, making Appellants' Brief due on June 17, 2007. Accordingly, this Brief is timely filed.

The Board of Appeals and Interferences has jurisdiction over this appeal pursuant to 35 U.S.C. §134.

Provided herewith is an authorization to charge the amount of $250.00 to cover the fee required under 37 C.F.R. §41.20(b)(2) for filing Appellants' Brief. In the unlikely event that the fee transmittal or other papers are separated from this document and/or other fees or relief are required, Appellants petition for such relief, including extensions of time, and authorize the Commissioner to charge any fees under 37 C.F.R. §§ 41.20(b)(2), 1.16, 1.17 and 1.21 which may be required by this paper, or to credit any overpayment, to Deposit Account No. 50-0815, order number STAN-276.

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REAL PARTY IN INTEREST (i)

The inventors named on this patent application assigned their entire rights to the invention to the Board of Trustees of the Leland Stanford Junior University. The real party in interest in this application is the Board of Trustees of the Leland Stanford Junior University.

RELATED APPEALS AND INTERFERENCES (ii)

There are currently no other appeals or interferences known to Appellant, the undersigned Appellant's representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

STATUS OF THE CLAIMS (iii)

Claims 1-9, 15, and 17-19 are pending and are appealed. Claims 10-14 and 16 are canceled.

This application was filed on November 13, 2003, and claims benefit of priority to U.S. Provisional Patent Application No. 60/426,677, filed November 15, 2002. Claims 1-14 were originally filed on November 13, 2003. In an amendment, filed on June 8, 2005 and responsive to the February 28, 2005 Office Action, claims 1, 5, and 6 were amended; and claims 15-18 were added. The June 8, 2005 amendments were entered. In an amendment, filed on January 13, 2006 and responsive to the August 25, 2005 final Office Action, claims 10-14 were canceled, and claim 19 was added. The January 13, 2006 amendments were entered; and as a result, claims 1-9 and 15-19 were pending. In an amendment, filed on August 2, 2006 and responsive to the May 8, 2006 Office Action, claim 16 was canceled, and claims 1 and 5 were amended. The August 2, 2006 amendments were entered; and as a result, claims 1-9, 15, and 17-19 were pending. IN an amendment, filed on January 18, 2007 and responsive to the October 18, 2006 final Office Action, claim 1 was amended. An Advisory Action issued on February 21, 2007. The February 21, 2007 Advisory Action indicated that the January 18, 2007 amendment would be entered. As a result of the amendments discussed above, claims 1-9, 15, and 17-19 remain pending.

All of the pending claims 1-9, 15, and 17-19 shown in the Claims Appendix remain pending, rejected, and appealed here.

STATUS OF AMENDMENTS (iv)

During the course of prosecution, the above-noted amendments were made. As noted above, February 21, 2007 Advisory Action indicated that the January 18, 2007 amendment would be entered. As such, the January 18, 2007, as well as all previous claim amendments, have been entered.

As such, there are no outstanding claim amendments; and claims 1-9, 15, and 17-19 as shown in the Claims Appendix are the claims of record, are pending, and are appealed here.

SUMMARY OF THE CLAIMED SUBJECT MATTER (v)

Appellants note that throughout this Appeal Brief, reference is made to the Substitute Specification and Substitute Drawings filed on June 14, 2004.

The instant application provides a method for detecting asymmetric dimethylarginine (ADMA) in a biological sample that may contain, in addition to ADMA, symmetric dimethylarginine (SDMA) and/or arginine. Substitute Specification, paragraphs 0029 and 0030.

Elevated ADMA levels have been observed in various pathological conditions, e.g., hypertension, dyslipidemia, hyperglycemia, hyperhomocysteinemia, and renal failure. Substitute Specification, paragraph 0004. Detection of ADMA levels in a sample, e.g., a biological sample, thus has clinical importance. Substitute Specification, paragraph 0004. Current methods of detecting ADMA suffer from low efficiency and sensitivity, primarily due to the difficulty of distinguishing ADMA from SDMA and from arginine; this difficulty is due to the fact that ADMA, SDMA, and arginine are chemically similar. Substitute Specification, paragraph 0031.

The instant invention as claimed provides a method of detecting ADMA in a sample comprising ADMA and at least one of SDMA and arginine. Claim 1; and Substitute Specification, paragraphs 0029 and 0030. The method generally involves contacting the sample with an α-dicarbonyl compound; the α-dicarbonyl compound modifies guanidino nitrogens of SDMA and arginine, but does not modify ADMA.

Claim 1, Substitute Specification, paragraphs 0031 and 0032. Modified SDMA and modified arginine are readily distinguishable from ADMA; thus, ADMA can be more accurately detected in a sample. Claim 1, Substitute Specification, paragraph 0032.

Various α-dicarbonyl compounds can be used to react with and modify SDMA and arginine that may be present in a sample. Claims 2 and 15; and Substitute Specification, paragraphs 0033-0036. To avoid possible modification of the α-amino group of ADMA, SDMA, and arginine, the α-amino group can be modified before the reaction with the α-dicarbonyl compound. Claims 3, 4, and 18; and Substitute Specification, paragraphs 0037 and 0044-0046.

Following modification of any SDMA and arginine which may be present in a sample that comprises ADMA, the ADMA is detected, using any of a variety of detection methods. Claims 1 and 5-9; and Substitute Specification, paragraphs 0047-0071.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL (vi)

There are two issues on appeal, as follows:

I. WHETHER CLAIMS 1-9, 15, AND 17-19 COMPLY WITH THE ENABLEMENT REQUIREMENT OF 35 U.S.C.§112, FIRST PARAGRAPH; AND

II. WHETHER CLAIMS 1-9, 15, AND 17-19 ARE INDEFINITE UNDER 35 U.S.C. §112, SECOND PARAGRAPH

ARGUMENTS (vii)

The arguments portion of this Brief is divided into two sections. The first section describes Appellants' understanding of the Examiner's rejections. The second section specifically addresses the issues outlined above.

With respect to the rejection under 35 U.S.C.§112, first paragraph, and with respect to the rejection under 35 U.S.C.§112, second paragraph, as set forth in October 18, 2006 final Office Action, claims 1-9, 15, and 17-19 are argued as a group and stand or fall together.

Rejection of claims 1-9, 15, and 17-19 under 35 U.S.C. §112, first paragraph

Claims 1-9, 15, and 17-19 were rejected under 35 U.S.C. §112, first paragraph, as allegedly failing to comply with the enablement requirement.

In support of this rejection, the Examiner argued that:

> Applicants' specification does not describe a two-step method comprising the steps of: a) contacting a sample with an α-dicarbonyl compound, followed by b) detecting ADMA in the sample. Applicants' specification does not describe the exact experimental conditions for performing said two-step method comprising the steps of: a) modifying SDMA and arginine, followed by b) detecting ADMA in the sample. Applicants' specification does not describe the exact reaction conditions for reacting a sample with an α-dicarbonyl compound, resulting in detectable ADMA. Applicants' specification does not describe a detecting means capable of detecting ADMA in the product of a reaction between a sample with an α-dicarbonyl compound. Other than phenylglyoxal derivatives (see Figs. 2 and 4), Applicants' specification does not contemplate any other "modified SDMA" derivatives or "modified arginine" derivatives.

October 18, 2006 final Office Action, page 3.

The Examiner concluded that the quantity of experimentation needed to perform the claimed two-step method of detecting ADMA is undue.

The rejection of claims 1-9, 15, and 17-19 under 35 U.S.C. §112, first paragraph is in error.

Compliance with the enablement requirement under 35 U.S.C.§112, first paragraph

To comply with 35 U.S.C. § 112, first paragraph, a specification need only enable a skilled artisan to make and use the claimed invention without undue experimentation. A specification complies with the statute even if a reasonable amount of experimentation is required, as long as the experimentation is not "undue." One way to determine if undue experimentation is required is to analyze the subject specification in

light of the *Wands* factors:[1] (1) the breadth of the claims, (2) the nature of the invention, (3) the state of the prior art, (4) the predictability or unpredictability of the art, (5) the quantity of experimentation necessary, (6) the relative skill of those in the art, (7) the amount of direction or guidance presented, and (8) the presence or absence of working examples. All of the factors need not be reviewed when determining whether a disclosure is enabling.[2]

Appellants submit that when evaluated in view of the relevant *Wands* factors, the specification enables one of skill in the art to practice the subject invention without undue experimentation.

The specification provides ample guidance.

The specification provides ample description of how to modify the guanidino nitrogens of SDMA and arginine.

The Examiner stated that the specification does not describe the exact experimental conditions for modifying SDMA and arginine.

However, the instant specification provides ample description of experimental conditions for performing the claimed method, involving contacting a sample with an α-dicarbonyl compound, where the α-dicarbonyl compound modifies guanidino nitrogens of SDMA and guanidino nitrogens of arginine, to produce modified ADMA and modified arginine, and detecting ADMA.

- The specification provides a list of suitable α-dicarbonyl compounds. Substitute Specification, paragraphs 0033-0035.
- The specification describes suitable concentrations of the α-dicarbonyl compound. Substitute Specification, paragraph 0038.
- The specification provides suitable reaction times and temperatures for the reaction between the α-dicarbonyl compound and the SDMA and/or arginine. Substitute Specification, paragraphs 0039 and 0042.

1 *In re Wands* 8 USPQ2d 1400 (Fed. Cir. 1988)
2 See *Amgen, Inc. v. Chugai Pharm. Co.*, 927 F.2d 1200, 1213, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991).

- Exemplary reaction conditions are also described. Specification, paragraph 00103.

Thus, given the guidance in the specification, those skilled in the art could readily carry out a method as claimed.

The specification provides ample description of methods for detecting ADMA.

The Examiner stated that the specification does not describe a detecting means capable of detecting ADMA.

However, the specification describes **at least four** methods of detecting ADMA. Substitute Specification, paragraphs 0047-0071. These methods include HPLC, capillary electrophoresis, immunoassays, and liquid chromatography-tandem mass spectrometry.

- **HPLC** As discussed in the specification, HPLC methods for detecting ADMA were known in the art. Substitute Specification, paragraph 0048. The instant specification provides a detailed description of an exemplary method of using HPLC to detect ADMA in a sample. Substitute Specification, paragraphs 0049-0054. The specification provides literature references that describe use of HPLC to detect ADMA. The literature references include: Teerlink et al. (Apr., 2002) *Anal. Biochem.* 303:131-137; Dobashi et al. (Jan., 2002) *Analyst* 127:54-59; Pi et al. (2000) *J. Chromatogr. B. Biomed. Sci. Appl.* 742:199-203; Chen et al. (1997) *J. Chromatogr. B. Biomed. Sci. Appl.* 692:467-471; Anderstam et al. (1997) *J. Am. Soc. Nephrol.* 8:1487-1442; and Pettersson et al. (1997) *J. Chromatogr. B. Biomed. Sci. Appl.* 692:257-262. Substitute Specification, paragraph 0048. Thus, as is evident from the specification and from the literature, those skilled in the art knew how to use HPLC to detect ADMA as of the November 15, 2002 priority date of the instant application.
- **Capillary electrophoresis** As discussed in the specification, capillary electrophoresis methods of detecting ADMA were known in the art. Specification, paragraphs 0056-0057. The specification provides literature references that describe use of capillary electrophoresis to detect ADMA, including Causse et al. (2000) *J. Chromatogr. B. Biomed. Sci. Appl.* 741:77. Substitute Specification, paragraph 0057. Thus, as is evident from the specification

and from the literature, those skilled in the art knew how to use capillary electrophoresis to detect ADMA as of the November 15, 2002 priority date of the instant application.

- **Immunoassays** As described in the instant specification, immunoassays can be designed that take advantage of the ability to distinguish modified SDMA and modified arginine from ADMA. Substitute Specification, paragraphs 0058-0069. Design and execution of such immunoassays is well within the skill level of those in the art.
- **Liquid chromatography-tandem mass spectrometry** As discussed in the specification, liquid chromatography-tandem mass spectrometry methods of detecting ADMA were known in the art. Substitute Specification, paragraph 0070. The specification provides a literature reference for use of liquid chromatography-tandem mass spectrometry to detect ADMA: Vishwanathan et al. (2000) *J. Chromatogr. B. Biomed. Sci. Appl.* 748:157-166. Substitute Specification, paragraph 0070. Thus, as is evident from the specification and from the literature, those skilled in the art knew how to use liquid chromatography-tandem mass spectrometry to detect ADMA as of the November 15, 2002 priority date of the instant application.

The specification contemplates use of any of a variety of α-dicarbonyl compounds, and provides a number of such compounds.

The Examiner stated that, other than phenylglyoxal derivatives, Applicants' specification does not contemplate any other modified SDMA derivatives or modified arginine derivatives.

This is not correct. As noted above, the specification provides a list of suitable α-dicarbonyl compounds. Substitute Specification, paragraphs 0034 and 0035. For example, the specification states that suitable α-dicarbonyl compounds include dialdehydes, ketoaldehydes, and diketones. Substitute Specification, paragraph 0034. The specification provides a non-limiting list of suitable α-dicarbonyl compounds, e.g., biacetyl, pyruvic acid, glyoxal, methyglyoxal, deoxyosones, 3-deoxyosones, malondialdehyde, 2-oxopropanal, phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione. Substitute Specification, paragraph 0034. Detailed conditions are provided for carrying out the reaction. Substitute Specification, paragraphs 0038-0039. Detailed conditions are provided for the α-dicarbonyl compound

phenylglyoxal. Substitute Specification, paragraph 00103. Phenylglyoxal is but one example of a suitable α-dicarbonyl compound that can be used.

The claims are not unduly broad.

The Examiner stated:

> Examiner has uncovered 242,619 searchable compounds falling within Applicants' definition of an "α-dicarbonyl" compound. Applicants' method does not enable a single one of these 242,619 compounds in the claimed two-step method.

February 21, 2007 Advisory Action, page 2.

However, the fact that there may be numerous compounds that are encompassed by "α-dicarbonyl compound" does not, by itself, indicate that the claimed method is not enabled. The claims recite use of an α-dicarbonyl compound that has the ability to modify guanidino nitrogens of SDMA and guanidino nitrogens of arginine. An α-dicarbonyl compound that does not modify guanidino nitrogens of SDMA and guanidino nitrogens of arginine is not encompassed by the claims.

Furthermore, while there may be some α-dicarbonyl compounds within the genus of α-dicarbonyl compounds, the courts have clearly taught that even in unpredictable arts the specification does not have to disclose every species of a genus that would work and every species that would not work.

The court has very clearly explained[3]:

> "To require such a complete disclosure would apparently necessitate a patent application or applications with thousands of catalysts....More importantly, such a requirement would force an inventor seeking adequate patent protection to carry out a prohibitive number of actual experiments. This would tend to discourage inventors from filing patent applications in an unpredictable area since the patent claims would have to be limited to those embodiments which are expressly disclosed. A potential infringer could readily avoid literal infringement of such claims by merely finding another analogous catalyst complex which could be used ...."

Because every species in a genus does not have to be tested for a genus to be enabled, extensive

3 *In re Angstadt*, 190 USPQ 214, at 219 (CCPA 1976)

disclosure or guidance of the active species of a genus does not have to be provided for a genus of this scope to be enabled.

No undue experimentation would be required to practice the claimed invention.

As discussed above, the specification provides ample guidance for carrying out the claimed method. Furthermore, as noted above, the skill level in the relevant art is high. The courts have clearly taught that the fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation.

As the court explained[4]:

> "[A] considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed."

Practitioners in the chemical arts frequently engage in extensive modification of reaction conditions and complex and lengthy experimentation where many factors must be varied to succeed in performing an experiment or in producing a desired result. The Federal Circuit has found that such extensive experimentation is not undue.

The claimed methods recite contacting a sample with an α-dicarbonyl compound, where the sample comprises ADMA and at least one of SDMA and arginine, where the α-dicarbonyl compound modifies guanidino nitrogens of SDMA and guanidino nitrogens of arginine; and detecting ADMA. The only experiments, if any, that need be performed to enable the entire scope of the claim are those designed to determine conditions under which a given α-dicarbonyl compound modifies guanidino nitrogens of SDMA and guanidino nitrogens of arginine. Such conditions are determined through routine experimentation, typically employing nothing more than conducting assays under conditions disclosed in the specification. As such, any experimentation that may be required to carry out a claimed method would be routine. Since such experiments are routine in nature, no undue experimentation is required.

4 *In re Wands* 8 USPQ 2d at 1404

The Examiner stated:

> **At most, Applicants disclose a means for detecting a hypothetical "analyte" by HPLC *after* extracting an unidentified "sample" and an unidentified "internal standard", derivatizing with an unidentified o-phthaldialdehyde derivatization procedure, followed by derivatizing with a vague "α-dicarbonyl" derivatization procedure[2] (see paragraphs [0047] and [0048]). Given the aforementioned deficiencies in Applicants' disclosure, Examiner posits that, even with the help and knowledge of prior art teachings, the quantity of experimentation needed to detect ADMA in Applicants' claimed two-step method is undue.**

February 21, 2007 Advisory Action, page 2.

Paragraphs 0047 and 0048 in the above-cited remarks correspond to paragraphs 0051 and 0052 in the Substitute Specification. The Examiner appears to be of the opinion that paragraphs 0051 and 0052 of the Substitute Specification do not provide enablement for a claimed method. However, paragraphs 0049-0054 of the Substitute Specification provide a description of non-limiting examples of how a claimed method could be used, together with HPLC, to detect ADMA in a sample.

As discussed above, the specification provides ample description, elsewhere in the specification, of the reaction between an α-dicarbonyl compound and guanidino nitrogens of SDMA and arginine. The cited paragraphs provide a description of an exemplary method in which a sample is contacted with an α-dicarbonyl compound in a manner recited in step (a) of claim 1; and the ADMA is detected using HPLC.

The skill level of those in the relevant field was high as of November 15, 2002.

Those of ordinary skill in the field of chemistry are familiar with and routinely carry out chemical reactions in which conditions such as pH, concentrations of reactants, temperature, and the like, are modified. Modifications of conditions used in a method involving contacting a sample with an α-dicarbonyl compound, where the α-dicarbonyl compound modifies guanidino nitrogens of SDMA and guanidino nitrogens of arginine that may be present in the sample, are within the skill level of a person with an undergraduate degree in organic chemistry. Thus, regarding step (a) of claim 1, the level of skill in the art was sufficiently high as of November 15, 2002 that a person of ordinary skill in the art, given the guidance in the instant specification and the knowledge in the art, could readily practice the full scope of the claim without undue experimentation.

Furthermore, as noted above, methods of detecting ADMA -- such as HPLC, capillary electrophoresis, immunoassays, and liquid chromatography-tandem mass spectrometry – were familiar to those skilled in the art as of the November 15, 2002 priority date of the instant application. Thus, regarding step (b) of claim 1, the level of skill in the art was sufficiently high as of November 15, 2002 that a person of ordinary skill in the art, given the guidance in the instant specification and the knowledge in the art (e.g., the literature cited in the specification), could readily practice the full scope of the claim without undue experimentation.

The cited art does not support a conclusion of lack of enablement of the instant claims.

In the October 18, 2006 final Office Action, the Examiner asserted that the state of the prior art appears to recognize a high degree of unpredictability in the field of arginine derivatization. In support of this assertion, the Examiner cited the following art: 1) Baburaj et al. ((1994) *Biochim. Biophys. Acta* 1199:253; "Baburaj"); 2) Schwarzenbolz et al. ((1997) *Z. Lebensm. Unters. Forsch. A* 205:121-124; "Schwarzenbolz"); and Sopio and Lederer ((1995) *Z. Lebensm. Unters. Forsch.* 201:381-386; "Sopio").

Baburaj

The Examiner stated that Baburaj discovered two α-dicarbonyl compounds ("HOCGO" and "DMACGO") that produced unexpected, unpredictable results when used to derivatize arginine. The Examiner stated that the findings of Baburaj "suggest that the ability of α-dicarbonyl compounds to distinguish between ADMA-containing samples versus non-ADMA containing samples may be somewhat limited using fluorescence-based detection." October 18, 2006 final Office Action, page 4.

Baburaj discusses the use of the α-dicarbonyl compounds HOCGO and DMACGO as pH-, polarity-, and quencher-sensitive fluorescent reporters for proteins that can be targeted at reactive arginines. The usefulness of such compounds, according to Baburaj, is as arginine modifiers, and as fluorescent probes. Baburaj, Abstract and Discussion. Baburaj states that both HOCGO and DMACGO are chemoselective in responding toward arginine (Arg) side chain but not towards cysteine (Cys) or lysine (Lys) side chains. Baburaj, Abstract. There is no discussion in Baburaj of a method of detecting ADMA in a sample that

contains, in addition to ADMA, at least one of SDMA and arginine.

Baburaj provides an extensive description of reaction conditions for reacting HOCGO and DMACGO with arginines. Thus, if anything, Baburaj supports the enablement of the instant methods as claimed.

The Examiner stated that Baburaj discovered that HOCGO and DMACGO:

1) are capable of reacting with cysteine and lysine residues (in addition to arginine);
2) are extremely sensitive to variations in solvent pH and polarity;
3) are capable of reacting with samples that don't possess arginine; and
4) react with samples non-covalently.

October 18, 2006 final Office Action, page 4.

None of the above-noted points raised by the Examiner has any bearing on the enablement of the appealed claims. Each of these points is addressed below.

1) "HOCGO and DMACGO are capable of reacting with cysteine and lysine residues."

As discussed during prosecution and during the January 12, 2007 telephone interview, the possibility that HOCGO and DMACGO might be capable of reacting with cysteine and lysine residues has no bearing on a determination of whether the instant claims are enabled. **All that is required is that the α-dicarbonyl compound modify any SDMA and any arginine that may be present in the sample**. Baburaj shows that the two α-dicarbonyl compounds discussed therein can react with arginine. Baburaj states: "Both [HOCGO and DMACGO] respond to proteins and Arg residues and are likely to be involved considering that both [HOCGO and DMACGO] are chemoselective in neither responding to Cys nor Lys side chains under appropriate dilute conditions." Baburaj, page 262, column 2, second paragraph. Indeed, Baburaj characterizes possible reactions with Cys and Lys as "a rare eventuality that can be ignored." Baburaj, page 262, column 2, second paragraph. The possibility that an α-dicarbonyl compound might modify a cysteine or a lysine residue is irrelevant. Any side reactions that would modify cysteines or lysines would not be

expected to adversely affect modification step (a) or detection step (b). For example, ADMA does not include cysteine or lysine. As such, Baburaj does not lead to a conclusion of lack of enablement.

2. *"HOCGO and DMACGO are extremely sensitive to variations in solvent pH and polarity."*

The reported sensitivity of HOCGO and DMACGO to variations in pH and polarity is discussed in Baburaj as an advantage, as HOCGO and DMACGO, once bound to an arginine in a protein, are viewed as useful sensors of pH and polarity. Baburaj, Abstract; and Discussion. Furthermore, the reported sensitivity of HOCGO and DMACGO to variations in pH and polarity is discussed in the context of after these compounds are reacted with arginine. The reported sensitivity is not discussed in the context of the reaction between these compounds and arginine. Still further, the reported sensitivity is irrelevant to the operation of the instant methods as claimed. As noted above, all that is required is that the α-dicarbonyl compound modify any SDMA and any arginine that may be present in the sample (and not modify ADMA). The modified SDMA and modified arginine are readily distinguishable from ADMA.

3. *"HOCGO and DMACGO are capable of reacting with samples that don't possess arginine."*

Baburaj discusses the proteins examined in the study, and states that ovalbumin showed a negligible response to HOCGO in the enhancement mode, and an appreciable response in the quenching mode. Baburaj, page 263, column 1, first paragraph. Baburaj attributes this to the absence in ovalbumin of a modifiable arginine and of a non-polar site for probe binding. Baburaj, page 263, column 1, first paragraph. While such side reactions may be of importance in studies of protein function as described in Baburaj, any such side reaction is irrelevant to the enablement of the instant methods as claimed. As noted above, all that is required is that the α-dicarbonyl compound modify any SDMA and any arginine that may be present in the sample (and not modify ADMA).

4. *"HOCGO and DMACGO react with samples non-covalently."*

Baburaj discusses possible non-covalent interactions of HOCGO and DMACGO with proteins. Baburaj, page 263, column 1, second paragraph. While such side reactions may be of importance in studies of protein function as described in Baburaj, any such side reaction is irrelevant to the enablement of the

instant methods as claimed. As noted above, all that is required is that the α-dicarbonyl compound modify any SDMA and any arginine that may be present in the sample (and not modify ADMA).

The Examiner stated that the findings of Baburaj "suggest that the ability of α-dicarbonyl compounds to distinguish between ADMA-containing samples versus non-ADMA containing samples may be somewhat limited using fluorescence-based detection." October 18, 2006 final Office Action, page 4. However, as discussed amply above, in the instant specification, and during prosecution, ADMA is not modified with an α-dicarbonyl compound. As such, none of the four points raised in the final Office Action and discussed above have any bearing on the enablement of the appealed claims.

Schwarzenbolz

The Examiner stated that Schwarzenbolz teaches that, under certain experimental conditions, the α-dicarbonyl compound glyoxal produces two arginine derivatives. The Examiner stated that "whether these and other derivatives are contemplated, and whether these derivatives are distinguishable from ADMA is not clear." October 18, 2006 final Office Action, page 5. The Examiner pointed to Figure 3 of Schwarzenbolz.

Schwarzenbolz discusses the reaction of glyoxal with arginine, and minor products that may be formed by the reaction of glyoxal with arginine. Figure 3 of Schwarzenbolz depicts products designated (3) and (4). Many chemical reactions will produce, in addition to a main product, one or more side products. The side products discussed in Schwarzenbolz are minor. Any side products that may be produced in such low quantities would not be expected to adversely affect modification step (a) or detection step (b). As such, Schwarzenbolz does not lead to a conclusion of lack of enablement.

Sopio

The Examiner stated that under certain experimental conditions, the α-dicarbonyl compound, deoxyosones, results in two tautomeric products; and stated that it is not clear whether these and other derivatives are contemplated, and whether such derivatives are distinguishable from ADMA. October 18, 2006 final Office Action, page 5.

Sopio discusses reaction of 3-deoxypentosulose with *N*-methyl- and *N,N*-dimethylguanidine as model reagents for protein-bound arginine and for creatine. Many chemical reactions will produce, in addition to a main product, one or more side products. The side products discussed in Sopio are minor. Any side products that may be produced in such low quantities would not be expected to adversely affect modification step (a) or detection step (b). As such, Sopio does not lead to a conclusion of lack of enablement.

Comments regarding the February 21, 2007 Advisory Action

The February 21, 2007 Advisory Action stated that the "cited prior art provide evidence that not all α-dicarbonyl compounds are useful in two-step methods"; and further stated:

> The cited prior art establishes the indiscriminant, unpredictable behavior of certain α-dicarbonyl compounds with respect to modification of both (a) analytes and (b) non-analytes. The cited prior art establishes that such indiscriminant, unpredictable behavior will negatively impact homogeneous assays, which require ascertainable and predictable modification of both (a) XOR (b).

February 21 Advisory Action, page 2.

First, as noted above, compliance with the enablement requirement of 35 U.S.C. §112, first paragraph, does not require that an applicant show that each and every species in a genus will work. Furthermore, as discussed in detail above, the cited art does not provide support for a conclusion of lack of enablement. None of the cited art casts doubt on the ability of an α-dicarbonyl compound to modify guanidino nitrogens of SDMA and arginine that may be present in a sample. None of the cited art casts doubt on the ability of a person of ordinary skill in the art, given the guidance provided in the instant application, to detect ADMA in a sample, following modification of guanidino nitrogens of SDMA and arginine that may be present in the sample.

Conclusion as to the rejection under 35 U.S.C.§112, first paragraph

The instant specification, combined with the knowledge and skill level of the person of ordinary skill in the art as of the November 15, 2002 priority date, provides ample description to enable the person of ordinary skill in the art to practice the claimed methods without undue experimentation. The specification provides ample guidance (e.g., the specification provides a description of how to modify the guanidino nitrogens of SDMA and arginine; the specification provides a description of methods for detecting ADMA;

the specification provides a number of α-dicarbonyl compounds; and the specification provides exemplary specific reaction conditions); the claims are not unduly broad; any experimentation that may be required to carry out a claimed method would be routine; and the skill level of those in the relevant field (e.g., organic chemistry) was high as of November 15, 2002. As such, no undue experimentation would be required to carry out an instant method as claimed. Furthermore, the cited art does not support a conclusion of lack of enablement of the instant claims.

Rejection of claims 1-9, 15, and 17-19 under 35 U.S.C. §112, second paragraph

Claims 1-9, 15, and 17-19 were rejected under 35 U.S.C. §112, second paragraph, as allegedly indefinite.

In support of this rejection, the Examiner argued that:

1) the phrase "said sample comprises ADMA and at least one of SDMA and arginine" is inconsistent with the preamble phrase "a sample comprising ADMA, SDMA, and arginine"; and

2) the recitation "said modified SDMA and said modified arginine are distinguishable" is indefinite. The Examiner stated that the "identity of object(s) and/or step(s), if any, required for performing distinguishing is not clear."

The amendment to claim 1, made in the response filed on January 18, 2007, which amendment was entered, adequately addresses issue (1), above. With respect to issue (2), the rejection of claims 1-9, 15, and 17-19 under 35 U.S.C.§112, second paragraph is in error.

1) The amendment to claim 1 adequately addresses issue (1).

Claim 1 is amended to recite "a sample comprising ADMA and at least one of SDMA and arginine" in the preamble. The phrase "said sample comprises ADMA and at least one of SDMA and arginine" is consistent with the preamble phrase "a sample comprising at least one of ADMA, SDMA, and arginine."

2) The recitation "said modified SDMA and said modified arginine are distinguishable" is clear.

To comply with the requirement of 35 U.S.C.§112, second paragraph, the claims must set out the subject matter **with a reasonable degree of clarity and particularity**. As set forth in MPEP §2173.02, in reviewing a claim for compliance with 35 U.S.C.§112, second paragraph, the examiner must consider the claim as a whole to determine whether the claim apprises one of ordinary skill in the art of its scope, in other words, whether the scope of the claim is clear to a person of ordinary skill in the relevant art.

As set forth in MPEP §2173.02, definiteness of claim language must be analyzed in light of:

a) the content of the disclosure of the patent application;
b) the teachings of the prior art; and
c) the claim interpretation that would be given by one possessing the ordinary level of skill in the art at the time the invention was made.

Claim 1 recites, in step (a), "wherein said modified SDMA and said modified arginine are distinguishable from ADMA." Step (a) recites reacting any SDMA or arginine that may be present in the sample with an α-dicarbonyl compound, resulting in modified SDMA and modified arginine, which, as discussed in the specification, are distinguishable from ADMA. The meaning of the phrase "wherein said modified SDMA and said modified arginine are distinguishable from ADMA" would be clear to those skilled in the art. As such, claim 1 need not be amended.

As discussed in the instant application, the structures of arginine, ADMA, and SDMA are very similar. Substitute Specification, paragraph 0031. The structures of arginine, ADMA, and SDMA are presented in paragraph 0031, and the fact that their structures are very similar is evident from the paragraph 0031. Reaction of arginine with an exemplary α-dicarbonyl compound is depicted in paragraph 0036; and reaction of SDMA with an exemplary α-dicarbonyl compound is depicted in paragraph 0037. The modified arginine depicted in paragraph 0036 and the modified SDMA depicted in paragraph 0037 have very different structures from ADMA. From the description in the specification, those of ordinary skill in the art would

have no trouble understanding the phrase "wherein said modified SDMA and said modified arginine are distinguishable from ADMA." As such, claim 1 is in compliance with the requirements of 35 U.S.C.§112, second paragraph.

Conclusion as to the rejection under 35 U.S.C.§112, second paragraph

Those of ordinary skill in the art, given the disclosure in the specification, would understand the phrase "wherein said modified SDMA and said modified arginine are distinguishable from ADMA." As such, claim 1 is in compliance with the requirements of 35 U.S.C.§112, second paragraph.

Appellants respectfully request that the rejections of claims 1-9, 15, and 17-19 under 35 U.S.C. §112, first paragraph and under 35 U.S.C. §112, second paragraph be reversed, and that the application be remanded to the Examiner with instructions to issue a Notice of Allowance.

Respectfully submitted,
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CLAIMS APPENDIX (viii)

1. (Previously presented) A method of detecting asymmetric dimethylarginine (ADMA) in a sample comprising ADMA and at least one of symmetric dimethylarginine (SDMA) and arginine, the method comprising the steps of:

a) contacting a sample with an α-dicarbonyl compound, wherein said sample comprises ADMA and at least one of SDMA and arginine, wherein said α-dicarbonyl compound modifies the guanidino nitrogens of SDMA and the guanidino nitrogens of arginine, producing modified SDMA and modified arginine, wherein said modified SDMA and said modified arginine are distinguishable from ADMA;

b) detecting ADMA in the sample.

2. (Original) The method of claim 1, wherein said α-dicarbonyl compound is phenylglyoxal.

3. (Original) The method of claim 1, further comprising the step of modifying the α-amino group of SDMA, ADMA, and arginine before the step of modifying the guanidino nitrogens of SDMA and the guanidino nitrogens of arginine.

4. (Original) The method of claim 3, wherein the α-amino group is modified with a dye that provides a detectable signal.

5. (Previously presented) The method of claim 1, wherein said detecting step comprises contacting the sample with an antibody that binds specifically to ADMA, to SDMA, or to both ADMA and SDMA, wherein said antibody does not bind to the modified SDMA.

6. (Previously presented) The method of claim 3, wherein said detecting step comprises contacting the sample with an antibody that binds specifically to the α-amino group-modified ADMA.

7. (Original) The method of claim 5, wherein the antibody is detectably labeled.

8. (Original) The method of claim 1, wherein said ADMA is detected by high performance liquid chromatography.

9. (Original) The method of claim 1, wherein said ADMA is detected by capillary electrophoresis.

10.-14. (Canceled)

15. (Previously presented) The method of claim 1, wherein the α-dicarbonyl compound is selected from biacetyl, pyruvic acid, glyoxal, methyglyoxal, deoxyosones, 3-deoxyosones, malondialdehyde, 2-oxopropanal, phenylglyoxal, 2,3-butanedione, and 1,2-cyclohexanedione.

16. (Canceled)

17. (Previously presented) The method of claim 1, wherein the sample is a biological sample.

18. (Previously presented) The method of claim 4, wherein the dye is a fluorophore.

19. (Previously presented) The method of claim 6, further comprising detecting one or more of modified SDMA and modified arginine, wherein said detection of one or more of modified SDMA and modified arginine comprises contacting the sample with an antibody that binds specifically to one or more of modified SDMA and modified arginine.

EVIDENCE APPENDIX (ix)

The following references were cited by the Examiner in the May 8, 2006 Office Action.

1) Baburaj et al. ((1994) *Biochim. Biophys. Acta* 1199:253;

2) Schwarzenbolz et al. ((1997) *Z. Lebensm. Unters. Forsch. A* 205:121-124; and

3) Sopio and Lederer ((1995) *Z. Lebensm. Unters. Forsch.* 201:381-386.

Copies of these references are provided herewith.

RELATED PROCEEDINGS APPENDIX (x)

There are currently no other appeals or interferences known to Appellant, the undersigned Appellant's representative, or the assignee to whom the inventors assigned their rights in the instant case, which would directly affect or be directly affected by, or have a bearing on the Board's decision in the instant appeal.

ELSEVIER

Biochimica et Biophysica Acta 1199 (1994) 253–265

BBA Biochimica et Biophysica Acta

HOCGO and DMACGO. Two coumarin derived α-dicarbonyls suitable as pH and polarity sensitive fluorescent reporters for proteins that can be targeted at reactive arginines

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Abstract

Two coumarin derived α-dicarbonyls, HOCGO and DMACGO, are presented as pH, polarity and quencher sensitive fluorescent reporters for proteins that can be targeted at reactive arginines. Both inactivate a number of enzymes that feature functionally critical and chemically susceptible arginyls. Both are chemoselective in responding towards Arg side chain but not towards Cys or Lys side chains under suitably dilute conditions. With $pK_{app} \approx 6.7$, HOCGO can serve as a pH sensor, while with $pK_{app} \ll 4.0$, DMACGO is better suited as a polarity sensor. A contrasting set of changes are manifest in the CGOs upon protein interaction that are either attributable to Arg modification or to the noncovalent probe associations with hydrophobic protein domains. DMACGO probes a single hydrophobic site on ovalbumin while HOCGO is largely unresponsive to this protein. Three to five arginyls are modified in HSA and BSA by HOCGO as well as DMACGO, while the latter also probes two hydrophobic sites on both these proteins. HOCGO modifies a single arginine in LDH active site, while its adducts with H_4 and M_4LDH isozymes titrate to the apparent pK_a of 7.8. Other proteins labeled with HOCGO or DMACGO reveal a number of variations that can furnish information about the microenvironment in the sites probed. The CGOs are thus potentially useful reporters of protein domains that feature reactive arginines. Suitable experimental condition are defined based on mechanistic considerations that may be used in applying the CGOs as Arg modifiers and as fluorescent probes.

Key words: Fluorescent probes; Arginine modification; Protein modification; Enzyme inhibitors

1. Introduction

The α-dicarbonyl reagents continue to remain in popular usage as protein modifiers. The reagents are often remarkably site selective, modifying those protein arginyls that are functionally critical, especially in enzyme active sites. Thus, arginyl residues represent attractive handles in labeling functionally important protein domains with fluorescent sensors [1–3], while α-dicarbonyls are the ideal prototypes based on which such sensors could be approached. In approaching fluorescent modifiers of arginine side chain, it was of particular interest to design them as the sensors of local charge and polarity.

The chemical susceptibility of a protein arginyl often correlates with its involvement as an anion recognition element [4–14]. Thus in their reactivity such arginyls manifest effects that appear to characterize the anion recognition centres. According to one popular suggestion, anion recognition centres are expected to share enhanced positive electrostatic potential, and this could be the causal factor in the reactive arginyls being associated with such centres [15]. Indeed, mechanistic considerations warrant a correlation between the side chain pKa of an arginine and its reactivity as a nucleophile [16,17], while electrostatic fields are known to

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Abbreviations: PGO, phenyl glyoxal; HOCGO, 7-hydroxy coumarinyl-3-glyoxal; DMACGO, 7-(dimethylamino) coumarinyl-3-glyoxal; *N*-Ac-Arg, N_α-acetyl-L-arginine; BGN, butylguanidine hydrochloride; BSA, bovine serum albumin; HSA, human serum albumin; ADH, alcohol dehydrogenase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PK, pyruvate kinase; AK, adenylate kinase; SOD, superoxide dismutase; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide (reduced).

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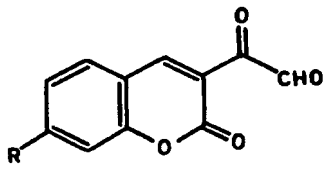
converge and concentrate in enzyme active site clefts [18–20] and can modulate functional group pK_a values in these clefts. However, other effects can also operate and do need to be considered as the determinants of such pK_a values in proteins [21]. Microenvironmental polarity is an important factor whose contribution in the prototropic equilibria at protein – water interface can not be ignored. The resolution of charge and polarity effects in the functional group pK_a values in proteins in general and in enzyme active sites in particular has, however, been a complex issue. There has been an increasing trend in applying the continuum electrostatic theory in computing the amino acid side chain pK_a values in proteins [20–23]. Because polarity effects are only indirectly treated in this theory, in the form of dielectric constant, its validity in correctly anticipating the pK_a values at protein-water interface remains uncertain. It may be noted that the pK_a values at micell-water interface do respond to the polarities [24] independent of the charge effects at this interface. The fluorescence probe method has been fruitfully applied in resolving the charge and polarity



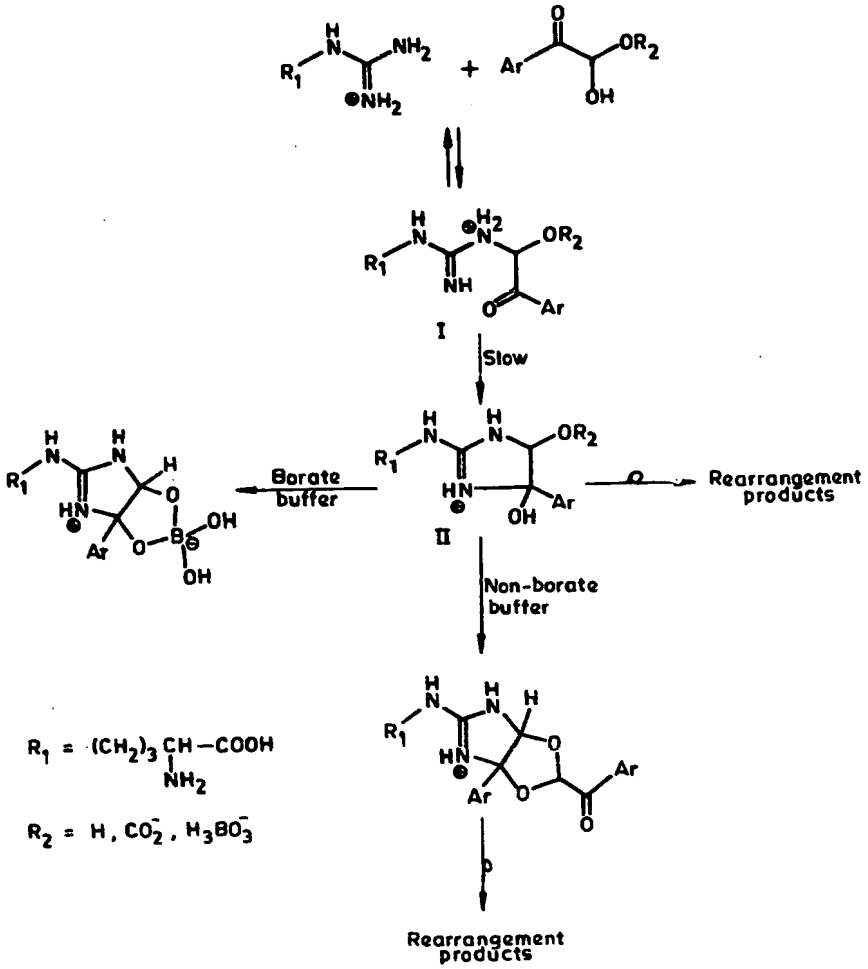
HOCGO; R = –OH

DMACGO; R = –N(Me)(Me)

Scheme 1.

effects at micell –water interface [24]. The method is also applicable to proteins, and charge and polarity sensors designed as arginine modifiers hold a particularly attraction for their potential in being applied to the anion recognition centres as a class.

Here we present HOCGO and DMACGO (Scheme 1) as arginine modifiers that can serve as the sensors of local charge and polarity in anion recognition centres based on their contrasting sensitivities to pH and sol-



Scheme 2.

vent polarities. Arginine-α-dicarbonyl reaction is a complex process [16], however, experimental conditions are defined, based on mechanistic considerations, that may be used in fruitfully applying the CGOs as microenvironmental sensors in characterizing the protein domains that feature reactive arginines.

2. Materials and methods

2.1. Materials

N-Ac-Arg, *N*-Ac-Cys, SeO_2, HSA, BSA, ovalbumin, rabbit muscle LDH, PK and AK, bovine heart MDH and bovine erythrocyte SOD were from Sigma Chemicals, St. Louis, MO, USA. The Sephadex gel was from Pharmacia, Uppsala, Sweden, while hen egg white lysozyme and yeast ADH were from SRL Chemicals, Bombay, India. All other chemicals were of reagent or analytical grade. Spectral grade solvents were used throughout the study and the buffers were prepared in triple distilled water.

2.2. Synthesis

HOCGO and DMACGO, obtained in good yield from the parent acylcoumarins [25] via SeO_2 oxidation, were crystallised from water and methanol, respectively, and furnished the following data:

HOCGO. m.p. 189–192°C, UV (MeOH): 427 nm ($\epsilon = 16\,100\ M^{-1}\ cm^{-1}$); PMR (DMSO-$d_6$), δ, 8.70 (1H, s, *ortho* to -COCHO), 7.82 (1 H, *d*, $J = 9$ Hz, *meta* to -OH), 6.88 (1H, *dd*, $J = 9$ Hz and 3 Hz, *ortho* to -OH and -H), 6.78 (1H, *d*, $J = 2.4$ Hz, *ortho* to -OH), 6.58 {2 H, *d*, $J = 9$ Hz, -CH(*OH*)$_2$} and 5.83 {1H, *d*, $J = 2.5$ Hz, C*H*(OH)$_2$}; *m/z* 218 (7% M^+).

DMACGO. m.p. 182–186°C, UV (MeOH): 432 nm ($\epsilon = 32\,000\ M^{-1}\ cm^{-1}$); PMR, (DMSO-$d_6$), δ, 9.84 and 5.84 {1H, 2 s, C*H*O and C*H*(OH)$_2$}, 8.64 and 8.59 {1H, 2 s, *ortho* to -CHO and -CH(OH)$_2$}, 7.76 and 7.69 {1H, 2*d*, $J = 9$ Hz, *meta* to -N(Me)$_2$}, 6.89 (1H, 2 *dd*, $J = 9$ Hz and 2.4 Hz, *ortho* to -N(Me)$_2$ and -H), 6.68 and 6.60 {1H, 2*d*, $J = 2.4$ Hz, *ortho* to -N(Me$_2$)} and 3.15 and 3.11 {6 H, 2 s, N*Me*$_2$)}; *m/z* 245 (8%; M^+).

2.3. Spectrophotometric measurements

All spectrophotometric studies were at 27 ± 0.1°C on a Shimadzu UV-265 instrument fitted with a thermostatted cuvette compartment and an external bath circulator. All fluorescence measurements were on a SPEX Fluorolog 2 series spectrofluorimeter, and were corrected for the lamp intensity and photomultiplier tube response.

2.4. Incubations

Unless otherwise indicated all incubations were at 27°C, in dark, at pH 7.5 (Hepes buffer, 50 mM) or 9.0 (Bicine or bicarbonate buffer, 50 mM).

2.5. Kinetic measurements

The arginine-glyoxal reactions were monitored by following the time-dependent decrease in the glyoxal concentration spectrophotometrically. In a typical experiment, a glyoxal dissolved in a requisite buffer was mixed with the 250 molar excess of a guanidine derivative and the decreasc in glyoxal concentration was monitored at an appropriate wavelength. The reaction was allowed to proceed to completion in order to correct for the absorbance due to the accumulated product. The pseudo-first order rate constants were calculated from the ln (glyoxal unreacted) vs. time plots.

2.6. Enzyme assays

All assays were in a total volume of 3 ml and were initiated by the addition of enzyme. (a) LDH activity was assayed in an assay system consisting of 85 mM potassium phosphate buffer (pH 7.5), 0.2 mM NADH and 2 mM sodium pyruvate, by monitoring the decrease in absorbance at 340 nm due to the oxidation of NADH in the presence of pyruvate [26]. (b) The MDH assay medium contained 6.3 mM L-malate, 2.7 mM NAD^+ and 0.11 M glycine adjusted to pH 10.0 with 5 M NaOH. The assay was monitored by noting the increase in absorbance at 340 nm due to the formation of NADH [27]. (c) Rabbit muscle PK activity was measured by coupling the production of pyruvate to the oxidation of NADH in 50 mM Hepes-KOH buffer (pH 7.5) containing 100 mM KCl, 2.5 mM PEP, 10 mM $MgSO_4$, 2 mM ADP, 10 units (U) of LDH and 1 U of PK. The absorbance at 340 nm was monitored for the oxidation of NADH (0.2 mM) [28]. (d) The activity of AK was measured in a coupled enzyme assay determining the production of ADP by means of PK and LDH. The assay was monitored in Hepes-KOH buffer (50 mM, pH 7.5) containing 80 mM KCl, 1.4 mM $MgCl_2$, 0.35 mM PEP, 0.8 mM ATP, 2.3 mM AMP, 5 U of LDH and PK each and 1 U of AK. The activity was monitored at 340 nm, owing to the NADH (0.18 mM) oxidation [29]. (e) The ADH activity was measured in glycine-NaOH buffer (20 mM, pH 8.9) containing 0.1 M sodium pyrophosphate, 1 mg/ml BSA and 0.3 mg/ml glutathione. The activity was monitored at 340 nm owing to the NAD^+ (2.5 mM) reduction in the presence of ethanol (0.5 M) [28]. (f) The assay for SOD was monitored in sodium carbonate (50 mM, pH 10.2) containing 100 μM EDTA. The activity was monitored

at 480 nm owing to the accumulation of adrenochrome via the auto-oxidation of epinephrine (0.3 mM) [30].

2.7. Enzyme inactivations

Enzymes were incubated with requisite glyoxals at specified concentration, in Hepes (50 mM, pH 7.5) or Bicine buffer (50 mM, pH 9.0). Aliquotes (100 μl) withdrawn at appropriate time intervals were added to the requisite assay cocktails (2.9 ml) to measure the absorbance changes. The stability of enzymes during the time course of experiment was verified using control assays devoid of the modifiers.

2.8. Protection experiments

The enzymes were incubated with natural ligands like NAD^+, NADH, oxamate, malate, pyruvate and citrate (20 mM each) in the presence of PGO, HOCGO or DMACGO (100 μM each) in Hepes buffer (10 mM) at pH 7.5, and aliquotes withdrawn at appropriate intervals were assessed for the residual enzyme activity. The control inactivations were conducted in the absence of the protecting reagents.

2.9. Fluorescence studies

Arginine specificity. The CGOs (5 μM each) were mixed with BGN, *N*-Ac-Cys or Boc-Lys (5 mM each) in Hepes buffer (50 mM, pH 7.5) and the reactions were monitored fluorimetrically for the product formation, setting the excitation wavelengths to 375 or 395 nm, and the emission wavelengths to 465 or 485 nm, for HOCGO or DMACGO, respectively.

Protein modifications. The CGOs (2 μM each) were incubated with BSA, HSA, lysozyme, ovalbumin, trypsin, ADH or chymotrypsin (20 μM each), for 5 h in Hepes buffer (50 mM) at pH 7.5. The reaction mixtures were then passed through a Sephadex G-25 column and the modified proteins were analyzed spectrofluorimetrically. Proteins treated as above were also analyzed fluorimetrically for the effect of variation in pH of the medium at a constant ionic strength of 0.1, which was set with NaCl.

HOCGO (100 μM) was incubated with H_4 and M_4 porcine LDH (10 μM) for 5 h in Bicine buffer (50 mM) at pH 9.0. The reaction was then passed through Sephadex G-25 column. The modified LDH was analyzed fluorimetrically for the effect of variation in pH of the medium at a constant ionic strength of 0.05, which was set with NaCl.

2.10. Scatchard experiments

The probe-protein titrations were conducted in dark by incubating varying concentration of the probe and the protein for 5 h in Hepes buffer (50 mM) at pH 7.5. In a typical experiment, a fixed concentration of the probe (say 10 μM) was titrated with varying concentration of the protein (say 0–30 μM), and a fixed concentration of the protein (say 10 μM) was titrated with varying concentrations of the probe (1–30 μM1), and the fluorescence was measured at the product emission. The protein titration data were plotted as ΔF^{-1} verses M^{-1} and the intercept on the *Y*-axis gave the value of limiting bound probe fluorescence (F_m). The stoichiometry (n) and the dissociation constants of probe binding (K_d) were determined from the bound probe (Pb) vs. the bound probe/free (Pb/Pf) plots [31].

Similar experiment was performed with HOCGO against LDH in Bicine buffer (50 mM) at pH 9.0. In the protein titration experiment, the probe concentration was 10 μM, while the protein concentration was varied in the range 2 to 5 μM. In the probe titration experiment, the protein concentration was 3 μM while the probe concentration was varied from 1 to 20 μM. The solutions were incubated for 10 h in dark at room temperature and analysed spectrofluorimetrically at an excitation wavelength of 380 nm.

2.11. Quenching studies

The CGOs (50 μM each) were incubated with BSA, HSA, lysozyme (150 μM each) and *N*-Ac-Arg (25 mM) for 3 h in Hepes buffer (50 mM) at pH 7.5. After equilibriation the modified proteins were passed through a Sephadex G-25 column to remove the unreacted probes. Aliquotes withdrawn from these solutions were examined for fluorescence in the presence of increasing concentration (0–0.6 M) of KI, CsCl and acrylamide.

2.12. Energy transfer experiments

The CGOs were incubated in increasing concentration (2–10 μM each) with a fixed concentration of BSA or HSA (10 μM 1 each) in Hepes buffer (50 mM, pH 7.5) for 3 h and the solutions were analyzed for fluorescence with λ_{ex} set to 290 nm.

3. Results

3.1. Synthesis and chemical characterization

HOCGO and DMACGO (Scheme 1) were obtained in good yield on oxidizing the corresponding acetophenones according to the method of Fodor and Kovacks [32]. Both are strongly chromophoric [DMACGO: λ_{max} (MeOH) 432 nm; ϵ 32000 $M^{-1}cm^{-1}$ and HOCGO: λ_{max} (MeOH) 427 nm; ϵ 16000 M^{-1} cm^{-1}], and re-

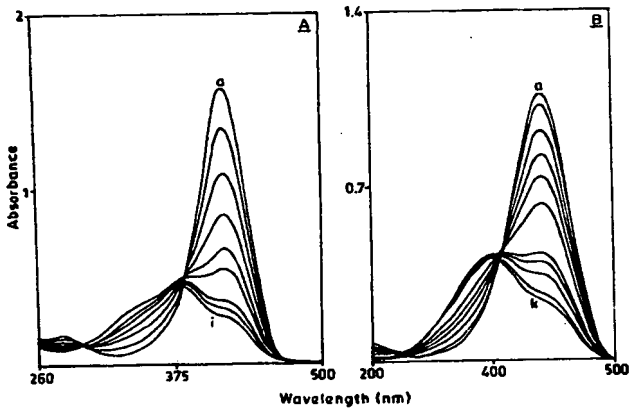


Fig. 1. Absorption spectra of HOCGO (Panel A) and DMACGO (Panel B) (trace *a* in each panel) and their response towards butylguanidine. The CGOs (50 μM each) were reacted with butylguanidine (25 mM) in Hepes buffer (50 mM, pH 7.5) and the solutions were examined for absorbance at increasing intervals.

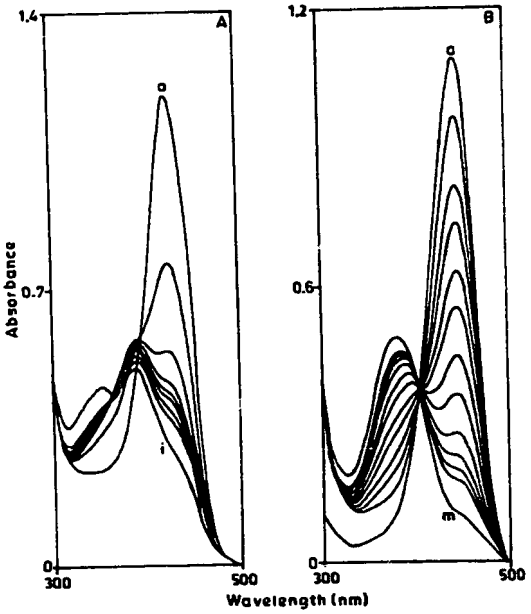


Fig. 2. Absorption spectra of HOCGO (Panel A) and DMACGO (Panel B) (trace *a* in each panel) and their response towards BSA. The CGOs (50 μM each) incubated with BSA (50 μM) in Hepes buffer (50 mM, pH 7.5) and the solutions were examined for absorbance at increasing intervals.

sponsive towards alkylguanidines. The absorption spectra of HOCGO and DMACGO are presented in Fig. 1 which also illustrates their spectral responses towards *N*-Ac-Arg. The diminution of long wavelength bands concomitant with the appearance of new bands at 375 and 395 nm, respectively, is consistent with the utilization of the ketonic groups of the CGOs due to arginine modification. Both reactions are characterized by the absence of a sharp isosbestic, however, no significant spectral changes occurred when the products were further incubated under these conditions for prolonged periods. Based on kinetics studies the CGOs compare well with PGO as *N*-Ac-Arg modifiers ($k_{obs} = 0.045$ min^{-1} for DMACGO, 0.031 min^{-1} for HOCGO and 0.038 min for PGO), while both are insensitive towards *N*-Ac-Cys and BOC-Lys under comparable conditions, showing no significant response on being incubated with these reagents (results not shown). Additional results corroborating the chemoselectivity of CGOs will be presented in a later section.

The CGOs are responsive towards proteins. Representative results from probe incubations with BSA are presented in Fig. 2. The observed changes, being similar to those in the *N*-Ac-Arg reaction, imply that protein arginyl(s) are modified. However, the HOCGO-BSA reaction is more complex than the HOCGO-*N*-Ac-Arg reaction as it reveals an even less specific isosbestic. As discussed later marked sensitivity of HOCGO in its ionization equilibrium appears to be the causal factor.

3.2. Enzyme inactivations

All enzymes examined here being sensitive to PGO are also inactivated by both the CGOs at pH 7.5 in Hepes buffer. All inactivations are essentially monoexponential indicating that either a single functionally critical arginine is involved or multiple arginines of comparable reactivity are involved in each case. The pseudo first order rate constants (k_{obs}) for the inactivations at equivalent reagent concentrations are listed in Table 1. DMACGO is a generally better inactivator than HOCGO and PGO which are comparable. The CGOs were effective inactivators of all these enzymes at pH 9.0 as well, however, appreciable inactivation rates were only realized in Bicine and bicarbonate but not in borate buffer (results not shown).

The enzymes LDH, MDH and PK were examined for protective effects of NADH, NAD^+, oxamate, malate, citrate and pyruvate against inactivations mediated by all the glyoxals. The results with LDH are shown in Fig. 3. Similar results were obtained with

Table 1
Relative reactivities of PGO, HOCGO and DMACGO (100 μM each) towards protein arginyls. The table shows pseudo-first order rate constants in the glyoxal mediated inactivation of LDH, MDH, ADH, PK, AK and SOD in Hepes buffer (50 mM, pH 7.5)

Enzymes	k_{obs} $(min)^{-1}$		
	PGO	HOCGO	DMACGO
LDH	0.0454	0.0454	0.0617
MDH	0.0101	0.0094	0.0359
PK	0.0071	0.0212	0.0200
AK	0.0587	0.1320	0.1320
ADH	0.0071	0.0030	0.0041
SOD	0.0110	0.0053	–

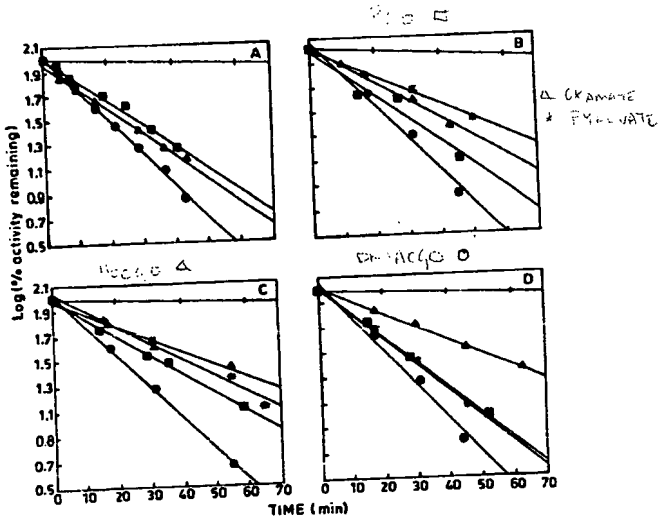


Fig. 3. Inactivation of LDH by PGO (■), HOCGO (▲) and DMACGO (●) (100 μM each) (Panel A) and its protection against PGO (Panel B), HOCGO (Panel C) and DMACGO (Panel D) (100 μM each) mediated inactivation by Oxamate (▲), Pyruvate (*) and NADH (■) (20 mM each) in Hepes buffer (50 mM) at pH 7.5. The time dependent decrease in LDH activity was measured as described in Section 2.

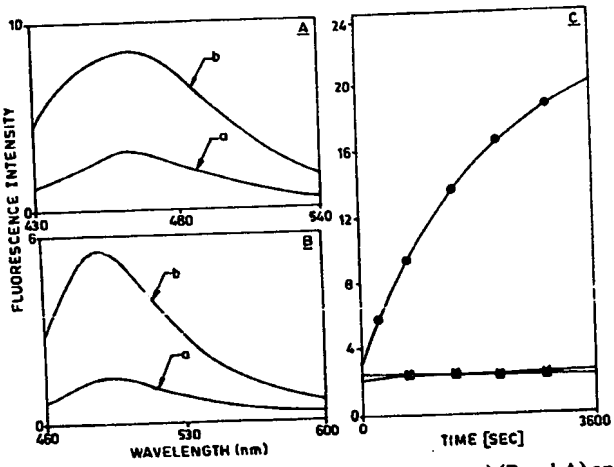


Fig. 4. Fluorescence spectra of HOCGO (λ_{ex} 375 nm) (Panel A) and DMACGO (λ_{ex} 395 nm) (Panel B) in the absence (Curve a) and presence of *N*-Ac-Arg (Curve b) and the time course of reaction of CGOs with BGN (●), *N*-Ac-Cys (■) and Boc-Lys (*) (Panel C). The CGOs (2 μM each) were reacted with BGN, *N*-Ac-Cys and Boc-Lys (10 mM each) in Hepes buffer (50 mM, pH 7.5) and fluorescence was monitored as a function of time, with λ_{ex} and λ_{em} being 375 and 462 nm, repectively, for HOCGO, and 395 and 485 nm, respectively, for DMACGO. The fluorescence intensity values are to an arbitrary scale.

other two enzymes as well (data not shown). Comparable effect of the active site ligands as protective agents against all the reagents implies that the same functionally critical arginyl(s) are susceptible in a given enzyme towards all the reagents.

3.3. Fluorescence measurements

The CGOs are appreciably fluorescent in methanol (DMACGO: $\lambda_{ex} = 432$ nm, $\lambda_{em} = 492$ nm; HOCGO: $\lambda_{ex} = 427$ nm, $\lambda_{em} = 465$ nm) and their quantum yields in this solvent, evaluated against perylene as the standard, are, approx., 0.1 and 0.9, respectively. Fig. 4, Panel A and B, illustrates the response of CGOs on being incubated with *N*-Ac-Arg. A 50 nm blue shift in excitation maximum occurs with both the probes, the emission wavelength are unaffected, however, emission intensities increase appreciably during the reaction. Thus CGOs can serve as arginine sensors and may be applied using the 'quenching' mode, following probe excitation (DMACGO $\lambda_{ex} = 445$ nm, and HOCGO $\lambda_{ex} = 425$ nm), or the 'enhancement' mode, following product excitation (DMACGO $\lambda_{ex} = 395$ nm, and HOCGO $\lambda_{ex} = 375$ nm). The responsiveness of CGOs towards BGN, *N*-Ac-Cys and BOC-Lys, monitored in the 'enhancement' mode, is illustrated in Fig. 4, Panel C. Both the CGOs only respond to BGN and are hence chemoselective.

3.4. Polarity and pH effects

The CGOs were examined for fluorescence in water-dioxan mixtures (Fig. 5). The fluorescence intensity of DMACGO increases while that of HOCGO decreases with decrease in solvent polarity. The net difference in intensity in pure water and pure dioxan is of the order of 27 in case of DMACGO and 37 in case of HOCGO. Decrease in solvent polarity also caused a blue shift in DMACGO emission, being 25 nm in going from pure water to pure dioxan, but had negligible effect on HOCGO emission wave length (results not shown). Given its poor solubility and uncertain stability

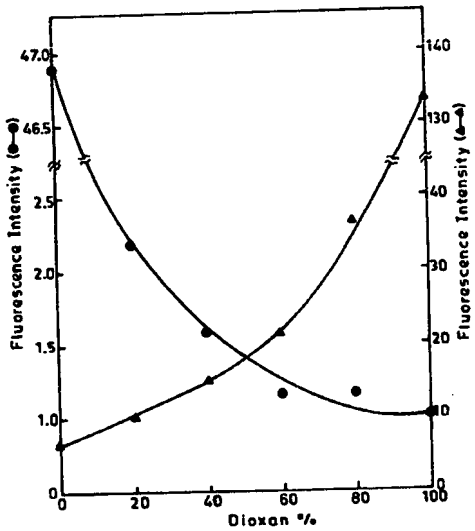


Fig. 5. Effect of increasing percentage of dioxan in water on fluorescence of HOCGO (λ_{ex} 425 nm) (●) and DMACGO (λ_{ex} 445 nm) (▲). The fluorescence intensity values are to an arbitrary scale.

no attempt was made to analyze the CGO-*N*-Ac-Arg adducts for their sensitivity towards solvent polarity.

The CGOs and their *N*-Ac-Arg adducts were examined for pH dependence. The results (Fig. 6, Panel A) establish that DMACGO and its *N*-Ac-Arg adduct are fully fluorescent down to about pH 4, and appreciable decrease in their fluorescence only occurs on further reducing the pH. HOCGO and its *N*-Ac-Arg adduct titrate in the higher pH range (Fig. 6 Panel B) giving p*K* values ≈ 6.7 and ≈ 7.6, respectively. HSA, BSA and the H_4 and M_4 isozymes of LDH modified with HOCGO were similarly examined. From the results with serum albumins (Fig. 6, Panel B) the p*K* of HSA-HOCGO adduct is comparable to that of HOCGO-*N*-Ac-Arg adduct, while that of modified BSA, being ≈ 8.3, is significantly higher. The LDH isozyme adducts (Fig. 6, Panel C) titrate to a pK_{app} that is comparable to that of HOCGO-*N*-Ac-Arg adduct.

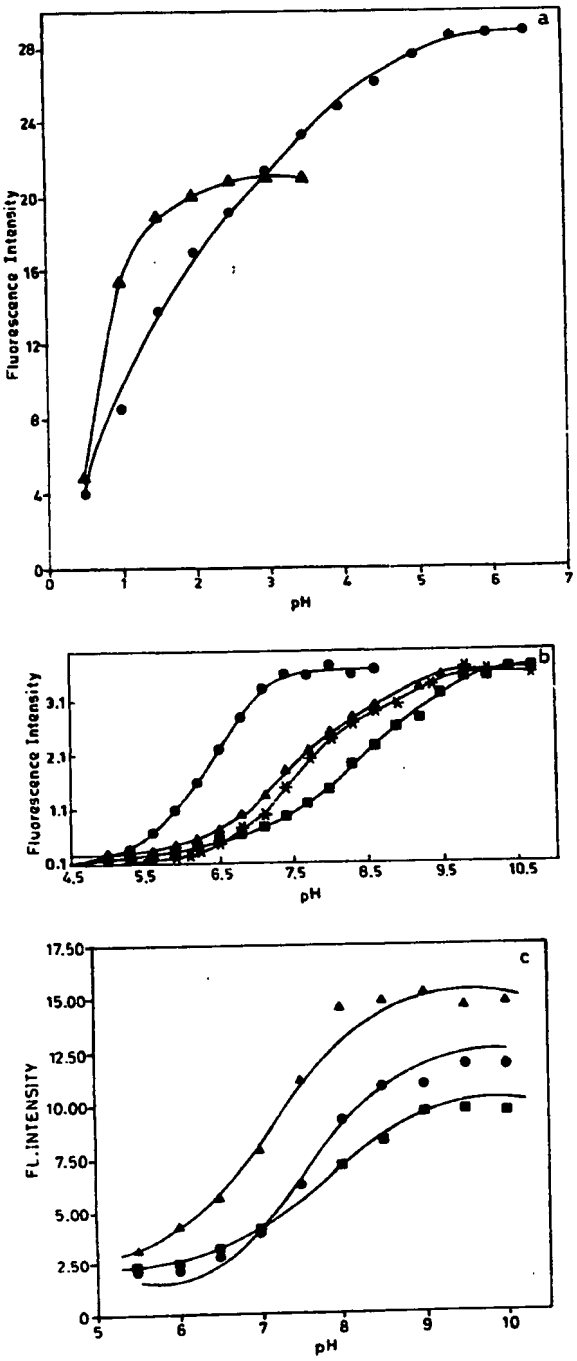


Fig. 6. Panel A: Dependence on pH of fluorescence of DMACGO (λ_{ex} 445 nm) (▲) and its product with *N*-Ac-Arg (λ_{ex} 395 nm) (●). The fluorescence intensity values are to an arbitrary scale. Panel B: Dependence on pH of fluorescence of HOCGO (λ_{ex} 425 nm) (●) and its products (λ_{ex} 375 nm) with *N*-Ac-Arg (▲), HSA (*) and BSA (■). The proteins (100 μM each) were incubated with HOCGO (50 μM) in Hepes buffer (50 mM, pH 7.5) for 5 h, desalted over Sephadex G-25 and aliquotes added to buffers of specified pH values. The fluorescence intensity values are to an arbitrary scale. Panel C: Dependence on pH of fluorescence of HOCGO (λ_{ex} 425 nm) (▲) and its products (λ_{ex} 380 nm) with H_4LDH (●) and M_4 LDH (■). LDH (10 μM) was incubated with HOCGO (100 μM) in Bicine buffer (50 mM, pH 9.0) for 5 h, desalted over Sephadex G-25 and aliquotes were added to buffers of specified pH values. The fluorescence intensity values are to an arbitrary scale.

3.5. Protein modifications

DMACGO and HOCGO were examined for responsiveness towards proteins in the 'quenching' as well as 'enhancement' mode. Marked variations were observed in the nature as well as time course of response depending upon the probe as well as the protein involved. Fluorescence spectra recorded after complete equilibriation of all the modification reactions are shown in Fig. 7. In the 'enhancement' mode (Panel C and D), all proteins elicit enhancements in emission intensities of both the probes, while some also manifest blue shifts in probe emission. In the 'quenching' mode (Panel A and B) most proteins elicit partial quenching of DMACGO fluorescence, while ovalbumin and ADH cause an extremely large enhancement in the emission intensity of this probe. Most proteins are partial quenchers of HOCGO fluorescence, while ADH and trypsin cause marginal enhancement in its emission intensity. Most probe-protein interactions revealed a slow time course, as would be expected in a chemical modification process, while those involving ovalbumin and ADH were rapid and apparently diffusion controlled.

The emission wavelengths of modified proteins are shown in Table 2. The values vary from protein to protein but the range of variation is generally small with HOCGO-protein adducts, excepting HSA- and BSA-adducts, in which case 18 and 12 nm blue shifts, respectively, are observed relative to the free probe

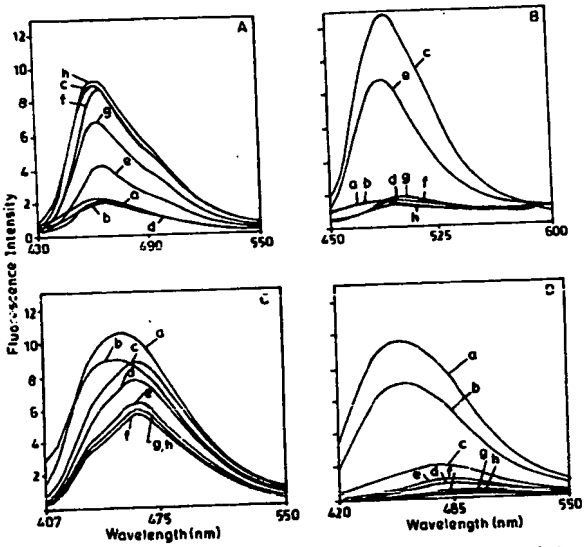


Fig. 7. Effect of proteins on fluorescence of HOCGO (Panel A and C) and DMACGO (Panel B and D), in the 'quenching' mode (Panel A and B) with λ_{ex} = 425 and 445 nm, respectively, for HOCGO and DMACGO and in the 'enhancement' mode (Panel C and D) with λ_{ex} = 375 and 395 nm, respectively, for HOCGO and DMACGO. (a) BSA, (b) HSA, (c) ADH, (d) lysozyme, (e) ovalbumin, (f) free probe, (g) chymotrypsin and (h) trypsin. The proteins (10 μM each) were incubated with CGOs (2 μM each) for 5 h in Hepes buffer (50 mM, pH 7.5), desalted over Sephadex G-25, and analyzed for fluorescence. The fluorescence intensity values are to an arbitrary scale.

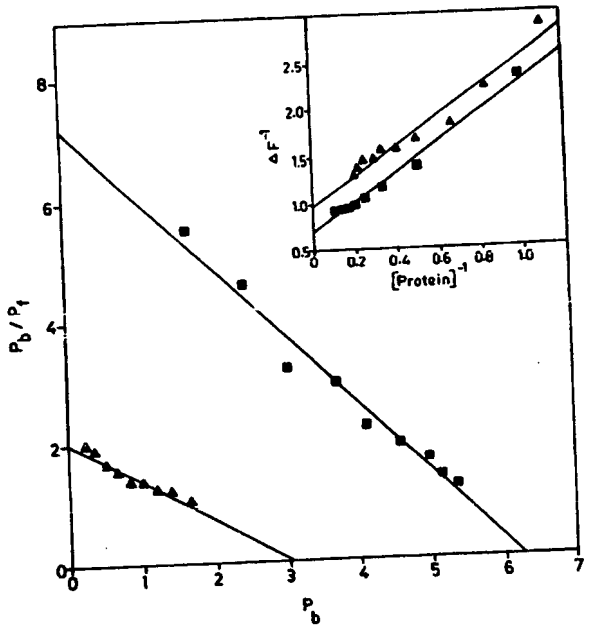


Fig. 8. Scatchard plots for HOCGO (▲) and DMACGO (■) reactions with BSA. The experimental procedure is described under *Materials and Methods*. The λ_{ex} and λ_{em} values were 375 and 450 nm, respectively, for HOCGO and 395 and 454 nm, respectively, for DMACGO. (Inset) Double reciprocal plots of changes in fluorescence intensity (ΔF) versus BSA concentration.

emission. The λ_{em} variations are more appreciable in case of DMACGO-protein adducts. Again the largest blue shift, of about 37 nm, is observed with the serum albumin adducts.

3.6. Scatchard experiments

The interactions of CGOs with serum albumins, ovalbumin and LDH were examined for concentration dependence in the 'enhancement' mode. The probe-serum albumin incubations were for 5 h to ensure complete equilibriation at even the lowest of probe-protein concentration. Representative results from HOCGO-BSA and DMACGO-BSA interactions, carried out at pH 7.5, are presented here. The variation of fluorescence intensity of the probes following protein titrations are presented as double reciprocal plots in Fig. 8, inset. Extrapolation of the plots to infinite protein concentrations furnish the limiting product fluorescence (F_{max}), and hence the product quantum yields in each case. The data from probe titration experiments are presented as Scatchard plots [31] (Fig. 8), which give the interaction parameters n (stoichiometry) and K_d (dissociation constant). Similar experiments were carried out at pH 9.0 in Bicine buffer to determine the n and K_d. The values obtained from several replicates at both the pH values are listed in Table 3. The ketone 3-acetyl-7-(dimethylamino)coumarin was examined for serum albumin interaction in Hepes buffer at pH 7.5. An enhancement of fluorescence noticed with this molecule could be attributed to the hydrophobic probe association. Titration experiments revealed two binding sites each for this molecule in HSA and BSA (results not shown). The observed stoichiometry in DMACGO-serum albumin interactions can therefore be attributed to noncovalent probe association as well as arginine modification.

The Scatchard experiment involving LDH-HOCGO in Bicine buffer at pH 9.0 (Fig. 9) reveals $n \approx 1$ and $K_d = 2.13 \cdot 10^{-3}$ M^{-1}, which can be attributed to arginine modification in this case.

Table 2
Emission wavelengths of CGOs and of CGO-protein adducts taken from the data in Fig. 7c and 7d. The excitation wavelengths are 375 and 395 nm, respectively, for the HOCGO- and DMACGO-protein adducts

HOCGO		DMACGO	
Reagent/protein	λ_{em} (nm)	Reagent/protein	λ_{em} (nm)
HOCGO	462	DMACGO	494
Lysozyme	461	Trypsin	490
Ovalbumin	461	Chymotrypsin	490
Chymotrypsin	461	*N*-Ac-Arg	487
N-Ac-Arg	461	Lysozyme	485
ADH	460	Ovalbumin	484
Trypsin	459	ADH	480
BSA	450	BSA	456
HSA	444	HSA	456

Table 3
Binding parameters in the interaction of CGOs with BSA and HSA. The incubations were in Hepes and Bicine buffer (50 mM each, pH 7.5 and 9.0, respectively). Each value is a mean ± S.D. from at least three independent experiments

Probe/protein	pH 7.5	
	n	K_d (M)
DMACGO/BSA	6.98 ± 0.72	$(0.90 \pm 0.03) \cdot 10^{-6}$
DMACGO/HSA	7.10 ± 0.70	$(1.86 \pm 0.0) \cdot 10^{-6}$
HOCGO/BSA	3.30 ± 0.23	$(1.09 \pm 0.25) \cdot 10^{-6}$
HOCGO/HSA	3.50 ± 1.00	$(0.98 \pm 0.22) \cdot 10^{-6}$
Probe/protein	**pH 9.0**	
	n	K_d (M)
DMACGO/BSA	8.60 ± 0.50	$(1.44 \pm 0.45) \cdot 10^{-6}$
DMACGO/HSA	5.10 ± 1.50	$(1.68 \pm 0.85) \cdot 10^{-6}$
HOCGO/BSA	7.00 ± 1.30	$(2.15 \pm 0.23) \cdot 10^{-6}$
HOCGO/HSA	6.00 ± 0.30	$(2.47 \pm 0.74) \cdot 10^{-6}$

The DMACGO-ovalbumin titrations were at pH 7.5 and monitored in the 'quenching' (probe excitation) mode. Much shorter incubation periods (≈ 15 min) were required for the equilibria to be established in this case. The results (not shown) gave the n and K_d values 0.97 and $2.46 \cdot 10^{-6}$ M, respectively.

3.7. Fluorescence quenching

The CGOs and their BSA-, HSA-, lysozyme- and *N*-Ac-Arg-adducts were examined for responsiveness towards some known quenchers. The cationic quencher Cs^{+1} and the neutral quencher acrylamide revealed no influence on either the CGOs or their adducts. The anionic quencher I^{-1} caused an appreciable quenching of fluorescence of both the probes and that of all their adducts. The results are presented in Fig. 10 as the Stern-Volmer plots. The plots, being linear, suggest that all the quenchings are of dynamic nature. HOCGO and its adducts are quenched to a greater degree (Panel B) than DMACGO and its adducts (Panel A). Also, the probe-*N*-Ac-Arg adducts are more strongly quenched than the probe-protein adducts. With the probe-protein adducts (Panel C) the I^{-1} quenching follows the order: lysozyme > BSA > HSA.

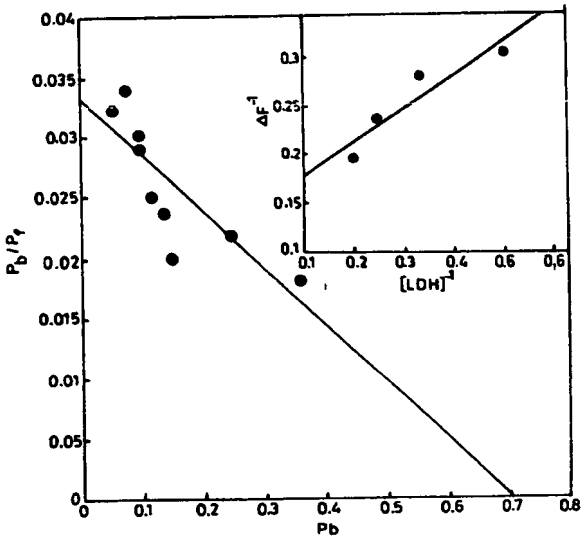


Fig. 9. Scatchard plot for HOCGO (●) reaction with LDH. The experimental procedure is described in Section 2. The λ_{ex} and λ_{em} values were 380 and 450 nm. (Inset) Double reciprocal plot of changes in fluorescence intensity (ΔF) versus LDH concentration.

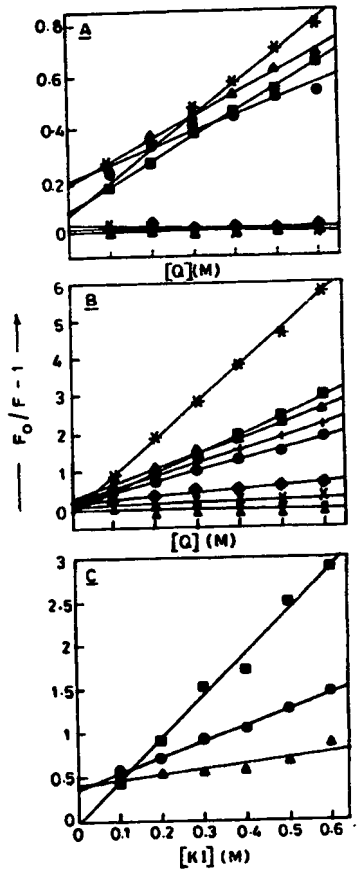


Fig. 10. Stern-Volmer plots for the quenching of fluorescence of CGOs and that of their products with *N*-Ac-Arg, BSA, HSA and lysozyme (DMACGO in Panel A and HOCGO in Panel B). KI/BSA (▲), KI/HSA (●), KI/*N*-Ac-Arg (*), KI/free probe (■), acrylamide/BSA (×) acrylamide/HSA (◆), acrylamide/*N*-Ac-Arg (+) and CsCl/*N*-Ac-Arg and CsCl/proteins (⧗). Panel C, KI mediated quenching of the fluorescence of HOCGO modified BSA (●), HSA (▲) and lysozyme (■).

3.8. Energy transfer

Tryptophan contributes appreciably in BSA and HSA fluorescence. The excitation spectra of CGO-serum albumin adducts overlap appreciably with the emission spectra of both these proteins following excitation at 290 nm. The modified proteins were thus

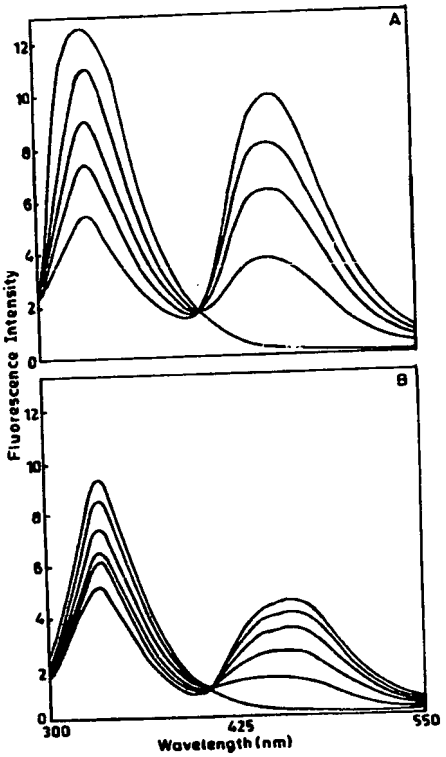


Fig. 11. Effect of HOCGO (Panel A) and DMACGO (Panel B) on BSA-tryptophan fluorescence. BSA (10 μM) was incubated with CGOs in increasing concentration (2–10 μM) in Hepes buffer (50 mM, pH 7.5) for 3 h. The excitation wavelength is 290 nm and the fluorescence intensity values are to an arbitrary scale.

examined for resonance transfer form tryptophan(s) to the probes and the results are presented in Fig. 11 (Panel A and B). Both probes quench BSA fluorescence in concentration dependent manner giving increasing emission at 465 nm. Similar results (not shown) were also obtained with HSA.

4. Discussion

Some of the general characteristics of HOCGO and DMACGO may be noted at the outset in appreciating the considerations that prompted their design. Both are arylglyoxals and could be expected, on this basis, to act as selective modifiers of arginine side chain. Both are built around coumarin nucleus, a well recognized fluorophore with wide ranging applications as a biochemical probe. With its phenolic auxochrome, HOCGO is a potential pH sensor applicable around the point of neutrality, while with its weakly basic dimethylamine auxochome unprotonable at neutral pH, DMACGO is a potential polarity sensor. Analogous molecules have been applied as sensors of charge and polarity at the micell-water interface [24]. The reactions of CGOs with arginine side chain would accompany a change in their chromophoric attributes, and hence the molecules could be expected to serve as the sensors of modifiable arginines as well.

4.1. Chemistry of arginine modification

Arginine modification is a complex process, susceptible to pH, buffer and ionic strength effects in its chemical course as well as kinetic profile. However, much has been learnt about the origins of these effects [16,17], hence better control over arginine modification can now be exercised based on proper choice of experimental conditions. Borate is a buffer commonly used in arginine modification that can simplify the reaction course furnishing the borate stabilized dicarbinolamine complexes. However, borate also complexes with the CGOs to largely inactivate them, and is hence unsuitable as the buffer in their application as arginine modifiers. Bicarbonate, the other buffer commonly used with α-dicarbonyls is known to activate them [16]. However, the alkaline pH can militate against the selectivity of modification process while it can also destabilize primary products in arginine modification causing them to rearrange [17]. Hence, Hepes and Bicine, being essentially inert towards arylglyoxals [16], were favored as the buffers in developing the CGOs as protein modifiers and fluorescent sensors.

4.2. CGOs as protein modifiers

In Hepes buffer at pH 7.5 the CGOs are nearly as reactive arginine modifiers as PGO. Both respond to proteins and Arg residues are likely to be involved considering that both are chemoselective in neither responding to Cys nor Lys side chains under appropriately dilute conditions. There are reports invoking Cys or Lys residues in the arylglyoxal mediated inactivations of some enzymes [33,34]. Such an eventuality can, in principle, arise if an enzyme active site features monodentate nucleophiles that can simultaneously attack the aldehyde as well as the ketonic centre in an arylglyoxal. In its effect on CGO fluorescence such a process may not be distinguishable from arginine modification. A degree of uncertainty will therefore persist regarding the residue identities when a protein is modified using the CGOs. However, this is a rare eventuality that can be ignored for the present. The reaction with arginine side chain alters the chromophoric attributes of the CGOs which are thus usable as the sensors of reactive arginines. However, being complex fluorophores, the CGOs also respond to other effects; HOCGO is extremely sensitive to pH variation while DMACGO is extremely sensitive to polarity variation. Hence fluorescence changes in CGOs upon protein interaction are not necessarily of chemical origin. As an extreme possibility, a CGO may respond to a protein without an arginine being involved. Both the CGOs

are prone to hydrophobic protein association and DMACGO in particular combines an appreciable nonpolar character with strong susceptibility as a polarity sensor. The notable enhancement of DMACGO fluorescence upon ovalbumin and ADH interaction is largely attributable to the noncovalent association of this probe with apolar domains in both the proteins. Both the CGOs manifest blue shifts in absorbance on reacting with arginine side chain. Hence the chemical reactions of CGOs are readily distinguishable from their noncovalent protein interactions.

Most proteins examined in this study reveal only limited number of modifiable arginines. Trypsin and chymotrypsin evoke a minimal response from both the probes, and are neither chemically modified nor noncovalently labelled to any appreciable degree. The negligible response of HOCGO to ovalbumin in the 'enhancement' mode but appreciable response in the 'quenching' mode can be attributed to the absence in this protein of a modifiable arginine but of a nonpolar site for probe binding. The protein evokes little response from DMACGO in the 'enhancement' mode, while the large enhancement in the 'quenching' mode suggests probe interaction without an arginine being involved. ADH manifests chemical modification in enhancing the HOCGO fluorescence, and noncovalent probe binding in enhancing the DMACGO fluorescence. Since both the CGOs inactivate ADH, the associated reaction is clearly reflected in the response of HOCGO towards this protein.

The marked responsiveness of both the CGOs towards BSA and HSA is attributable to the combined effect of probe binding as well as arginine modification. The coincidence of these effects is particularly evident in the minimal response of DMACGO to both these proteins in the 'quenching' mode. Reaction with arginine and noncovalent binding affect DMACGO fluorescence in the opposite sense and hence in the mutually canceling ways. The two effects coincide in the 'enhancement' mode, and explain the large enhancement in probe fluorescence in this mode. Possible association of DMACGO with two non arginine bearing sites on serum albumins is substantiated by the association of the ketonic precursor of DMACGO – *viz*, 3-acetyl-7-(dimethylamino)coumarin – with HSA as well as BSA, with the stoichiometry of two.

In assessing probe-protein stoichiometries, the molar fluorescence of specific protein-probe adducts need to be first determined. The underlying assumption in applying this approach is that all the adducts, should a protein be modified at more than one site, will have identical quantum yields. Considering their complexity as fluorophores this assumption is unlikely to be generally valid with the CGOs. Within this limitation, the probe-serum albumin stoichiometries at pH 7.5 are assessed as $\approx 3:1$ against HOCGO and $\approx 7:1$ against DMACGO. Assuming that DMACGO interacts with two non arginine sites on both the proteins, nearly five arginines are modified in both the proteins by DMACGO. At pH 9.0, two more arginyls appear to be modified by HOCGO in HSA as well as BSA, but not by DMACGO. The good concordance in five reactive arginines being detected at this pH in the two proteins by HOCGO as well as DMACGO is noteworthy considering the sharply contrasting properties of the two probes as fluorophores. The implication is that majority of the arginyls in serum albumins are indeed chemically refractory even under this nondenaturing however relatively harsh reaction condition. Considering that reactive arginines are generally associated with anion recognition centres, it is of interest to recall that both BSA and HSA are richly endowed with anion binding sites that include a number of topologically distinct fatty acid binding sites [35]. Presumably, the reactive arginines now detected in serum albumins are associated with one or more of these sites [36].

4.3. CGOs as enzyme inactivators

As enzyme inactivators, the CGOs have been found to compare generally well with PGO. Judged by the protective effects of active site ligands, the same arginyl(s) modified by PGO in an enzyme appear to be also susceptible towards the CGOs. Some of the enzymes examined in this study, especially LDH, AK and SOD, are modified by PGO at a single arginine [29,37–39], and exemplify the remarkable degree of site selectivity that α-dicarbonyls are reputed to display as protein modifiers. Positive electrostatic fields and diminished local polarities are possible factors that activate these arginyls in preference to many other arginyls that all these proteins feature on their solvent exposed surfaces. Thus the CGOs become attractive probes in examining such enzymes for their active site microenvironments.

4.4. CGOs as charge and polarity sensors

Fluorescent sensors of charge and polarity have been approached on three distinct principles. The earliest approaches led to probes such as ANS and prodan [40,41] characterized by marked differences in dipolar strengths in the ground and excited states, making their emission characteristics susceptible towards solvation effects. DMACGO and its arginine adducts share the electronic attributes of these classical polarity sensors. The variations noticed in λ_{em} values of DMACGO-protein adducts could be physically meaningful but are difficult to rationalize given the multiple site modifications and uncertain product identities in our study. Also polarization effects can manifest in probe-protein adducts which need to be resolved while

the ruler required in translating the observed λ_{em} values to local polarities needs to be elaborated. Meaningful applications of DMACGO are likely to be better realized by combining the use of polarization and life time measurements especially against proteins that are uniquely and selectively modified.

Fluorescent sensors of charge and polarity have also been designed as pH probes. It is recognized that, in general, the pKa of an ionizable group placed at the solvent-macromolecule interface can respond to charge as well as polarity on this interface that can be treated by the general relation [42]:

$$pK_{app} = pK_a - \frac{e\psi(o)}{2.3\,kT} + [\Delta pK_a^p]$$

HOCGO has the requisite attributes and its application as a pH sensor appears feasible given our results with serum albumins and with LDH. The different pK_{app} values observed for the HSA-HOCGO and BSA-HOCGO adducts are curious considering the homologous structure and function of these proteins, but difficult to rationalize, considering that multisite modifications are involved. The pK_{app} of H_4 and M_4 LDH adducts of HOCGO may more particularly correlate with the active site attributes in these isozymes because apparently a single arginine is uniquely modified in each one.

Responsiveness towards charged quenchers has been the other important approach in applying fluorescence molecules as charge sensors in membrane systems [43]. The CGOs are insensitive towards Cs^+ and acrylamide, but responsive towards I^- as the quencher. The differences noticed in I^- quenching of the probes bound to free arginine and to different proteins are of note, however, it remains to be ascertained whether these are of steric or electrostatic origin.

Lastly, the resonance transfer of energy observed between the probes and tryptophan residues in BSA makes CGOs promising tools as the distance rulers in protein studies.

4.5. Conclusion

HOCGO and DMACGO have been developed as potentially useful reporters of anion recognition centres in proteins. As fluorophores both are responsive towards arginines and sensitive towards pH, polarity and quencher effects. Appreciable variations are noticed in the fluorescence of CGO-protein adducts that reflect sensitivity of the probes towards local microenvironment. Suitable reaction conditions and approaches are defined in applying the CGOs as arginine modifiers and as the sensors of charge and polarity at the protein-water interface.

5. Acknowledgements

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6. References

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ORIGINAL PAPER

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On the reaction of glyoxal with proteins

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Abstract The reaction of arginine and arginine derivatives with glyoxal under mild conditions revealed the formation of a previously unknown amino acid, designated as "Glarg". ^{1}H-, ^{15}N- and ^{13}C-NMR analysis of the new compound elucidated its structure to be 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine. Experiments with solutions containing N^{α}-acetylarginine and glyoxal showed that "Glarg" is formed quickly under physiological conditions, but is labile at higher temperatures as well as at low pH values. After incubation of β-casein with glyoxal, the formation of protein-bound "Glarg" in enzymatic hydrolysates via amino acid analysis could be demonstrated. Due to the fast reaction of glyoxal with arginine residues, under physiological conditions, proteins may act as scavengers for glyoxal.

Key words Arginine · Glyoxal · Maillard reaction · Protein modification · 1-(4-Amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine

Introduction

An advanced Maillard reaction and carbohydrate degradation in alkaline as well as in acidic media gives rise to the formation of various α-dicarbonyl compounds. Among these, the presence of glyoxal was detected in reaction mixtures of carbohydrates and amines [1] and it is reported to be a major product of glucose degradation under oxidative conditions [2]. The detection of glyoxal in edible oil [3] as well as in fermented foods [4] shows that it is also formed as a product of lipid oxidation and of microbial fermentation. As demonstrated by amino acid analysis, α-dicarbonyls are highly reactive towards proteins, targeting lysine and arginine residues in particular [5, 6]. To date two protein-bound products of the reaction between α-dicarbonyls and amino acids – namely ornithinoimidazolinone 1 (Fig. 2), formed in the reaction between methylglyoxal and arginine residues, and pyrraline, originating from lysine and 3-deoxyhexosulose – have been detected in food [7, 8]. Compound 1 may undergo further oxidation to form the corresponding imidazolone [9]. A similar imidazolone structure was isolated from reaction mixtures of 3-deoxyhexosulose and N^{α}-benzoylarginine amide [10]. Using N^{α}-hippuryllysine as a model for protein-bound lysine, the formation of cross-linking imidazolium salts from both methylglyoxal and glyoxal has been described [11, 12]. For bovine serum albumin, the reaction of lysine residues with glyoxal to form N^{ε}-carboxymethyllysine or C2-imine cross-links respectively was observed [13].

In the present study we investigated the reaction of protein-bound arginine with glyoxal, and we elucidated a new arginine derivative by means of fast-atom bombardment-mass spectrometry (FAB-MS) and nuclear magnetic resonance (NMR).

Materials and methods

Chemicals. N^{α}-Acetyl-L-arginine, glyoxal and β-casein were obtained from Sigma (Deisenhofen, Germany). Pepsin and pronase were from Merck (Darmstadt, Germany).

The suspension of aminopeptidase M was from Boehringer (Mannheim, Germany). Prolidase was from Serva (Heidelberg, Germany). Hydrochloric acid was from J.T. Baker (Deventer, The Netherlands). Dowex AG 50W-X8 (200–400 mesh, H^{+} form) ion-exchange resin was from Bio-Rad (Munich, Germany). All other chemicals were from Merck.

Acidic deacetylation. N^{α}-Acetyl-L-arginine and glyoxal model systems contained 0.1 mmol/ml N^{α}-acetyl-L-arginine and varying amounts of glyoxal. They were deacetylated by addition of 400 µl of 4 M HCl to 400 µl of the incubation mixture and subsequent heating at 110 °C for 100 min. These hydrolysates were deluted 50-fold with buffer 1 (see

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Table 1 Nuclear magnetic resonance data of "Glarg". δ values are given relative to those of tetramethylsilane

^1H		^{13}C		^{15}N	
Atomic assignment	δ (ppm)	Atomic assignment	δ (ppm)	Atomic assignment	δ (ppm)
(C-2′)H_2	1,69	C-2′	23,20	(C-4′)NH_2	41,94
(C-3′)H_2	1,84	C-3′	26,87	N-3	83,72
(C-1′)H_2	3,65	C-1′	38,59	(C-2)NH_2	86,21
(C-4′)H	3,84	C-4	47,50	N-1	147,21
(C-4)H_2	4,16	C-4′	51,42		
COOH	8,50	C-2	158,62		
(C-4′)NH_2	8,61	COOH	170,54		
(C-2)NH_2	9,56	C-5	171,43		
(N-3)H	9,76				

amino acid analysis) and 40 µl aliquots were applied on the amino acid analyser.

Enzymatic hydrolysis. β-Casein samples were hydrolysed using four enzymes (pepsin, pronase E, aminopeptidase and prolidase) as described previously [14].

Amino acid analysis. This was performed using an Alpha Plus amino acid analyser (LKB Biochrom, Cambridge, UK), using a stainless steel column (150 × 4 mm, Alltech, Unterhaching, Germany) filled with ion-exchange resin DC4A-spec, lithium form (Benson, Reno, Nev., USA). The elution program started with buffer 1 (0.06 mol/l trilithiumcitrate, 0.1 mol/l LiCl, pH = 3.0, containing 25 ml/l 2-propanol) for 25.5 min at a column temperature of 32 °C. After this, the column was eluted with buffer 2 (0.06 mol/l trilithiumcitrate, 0.4 mol/l LiCl, pH = 3.2) for 10 min at 36 °C and 10 min at 50 °C. This was followed by buffer 3 (0.06 mol/l trilithiumcitrate, 0.8 mol/l LiCl, pH = 3.4) for 10 min at 65 °C and 10 min at 68 °C and buffer 4 (0.06 mol/l trilithiumcitrate, 1.4 mol/l LiCl, pH = 3.5) for 12 min at 80 °C and 24 min at 72 °C. Regeneration of the column was achieved by lithiumhydroxide (0.3 mol/l, 8 min, 80 °C), followed by equilibration with buffer 1 for 35 min at 80 °C and 10 min at 32 °C. The flow rate was at 20 ml/h. Amino acids were detected using ninhydrin [15], the detection wavelengths were set at 570 nm and 440 nm.

Synthesis of the imidazolidine 4 (Fig. 3). Arginine (1743 mg, 10 mmol) was dissolved in 100 ml water and 2 ml of 40% aqueous solution of glyoxal (15 mmol) was added drop by drop. After adjustment of the pH value to 7.4, the reaction mixture was kept at 37 °C for 4 h. This solution was evaporated under reduced pressure at room temperature to approximately 15 ml. After adjusting the pH to 2.0 with 1 M HCl, the solution was applied to a column (16 × 120 mm), filled with cation-exchange resin Dowex 50W-X8, that had previously been equilibrated with 75 ml of 2 M HCl and 25 ml water. The column was eluted with each 50 ml of 0.5 M HCl, 1 M HCl and 1.5 M HCl at a flow rate of 1 ml/min, finally with 150 ml of 2 M HCl at a flow rate of 0.8 ml/min. Fractions (5 ml) were collected and checked for ninhydrin reaction. Then, 10-µl aliquots of the ninhydrin-positive fractions were run on the amino acid analyser. Compound 4 was found in fractions 9–20 of the 2 M HCl eluate. These fractions were pooled and evaporated to dryness at room temperature, yielding 35 mg of an amorphous yellow powder.

NMR analysis. ^1H- and ^{13}C-NMR spectra (solvent [D_6]-DMSO, external standard tetramethylsilane) were recorded with a 600 AMX (600 MHz) instrument (Bruker, Rheinstetten, Germany).

Model mixtures. Solutions of 0.1 mmol/ml N^{α}-acetylarginine in PBS (294 mg Na_2HPO_4, 1358 mg NaCl and 86 mg KH_2PO_4 in 100 ml water, pH 7.4) with the desired equivalent (0.2, 0.1 or 0,05 mmol) of a 4% aqueous glyoxal solution were heated under varyious conditions (T = 30–80 °C, t = 0–96 h).

Incubation of β-casein with glyoxal. Solutions of β-casein (40 mg/ml, M_r = 24000) were mixed with varying amounts of 4% aqueous glyoxal solution. The additions were equivalent to 7.2 µmol, 3.8 µmol and 2.4 µmol respectively, corresponding to arginine/glyoxal ratios of 1:1, 2:1 and 3:1.

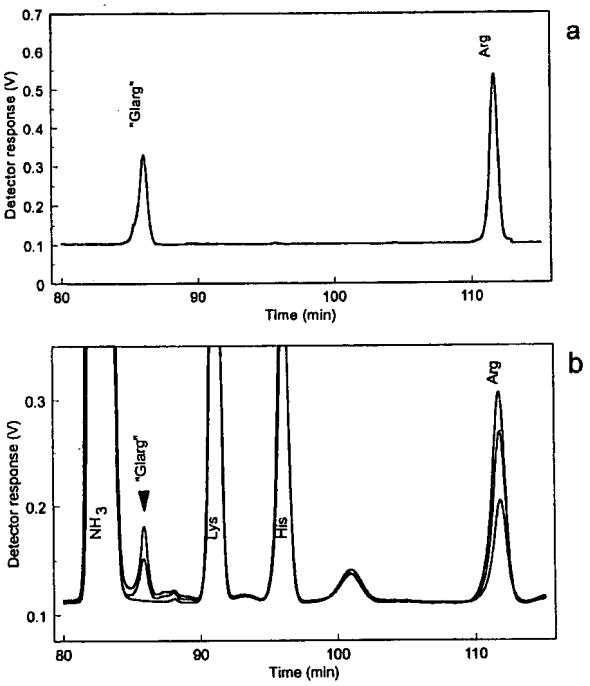


Fig. 1a, b Chromatograms (detail) of hydrolysates. **a** Incubation mixture of N^{α}-acetylarginine and glyoxal, mild acidic deacetylation. **b** Incubation mixtures of β-casein with varying glyoxal concentrations, enzymatic hydrolysis

Results and discussion

After incubation of N^{α}-acetylarginine with glyoxal in molar ratios of 1:1 at 80 °C, and subsequent acid hydrolysis, amino acid analysis showed a loss of arginine as well as the formation of a new ninhydrin-positive compound (Fig. 1a), designated "Glarg". In the chromatogram, "Glarg" eluted between ammonia and lysine (Fig. 1b). FAB-MS of isolated material revealed a pseudomolecular ion (MH^+) at m/z = 215.1, indicating the formation of a

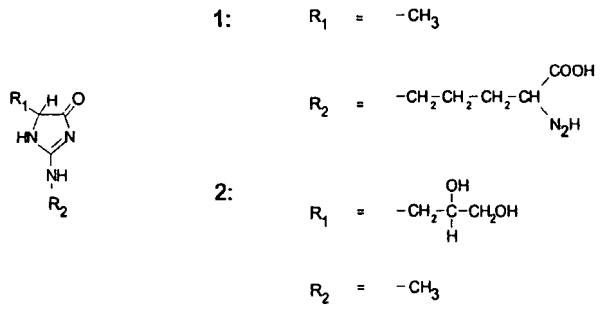
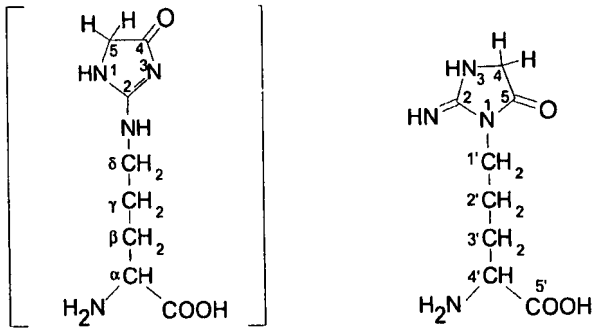


Fig. 2 *N*δ-(5-Methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine (1) and 2-methylamino-5-(2,3-dihydroxypropyl)-2-imidazolin-5-one (2)



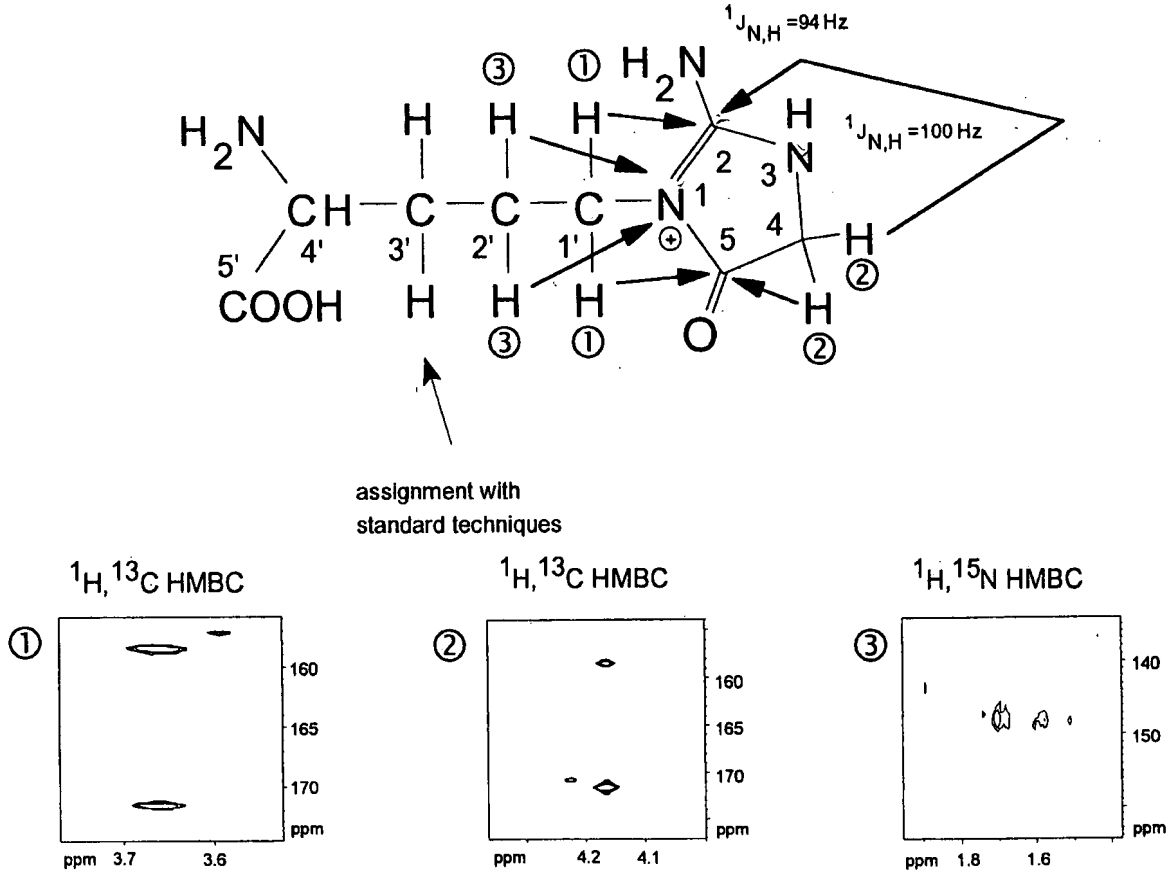
3 4

Fig. 3 *N*d-(5-Dihydro-4-oxo-imidazol-2-yl)-L-ornithine (1) and 1-(4-amino-4-carboxy butyl)-2-imino-5-oxo-imidazolidine (2)

compound with the empirical formula $C_8H_{14}O_3N_4$ (calculated M_r = 214.2), derived from a condensation reaction between one molecule each of arginine (M_r = 174.2) and glyoxal (M_r = 58.0) with loss of one molecule of water (M_r = 18.0).

For unequivocal structure elucidation, ^{1}H-, ^{13}C- and ^{15}N-NMR analysis was performed. Chemical shifts are listed in Table 1. There are several possible structures produced by condensation of arginine and glyoxal. As proposed in the literature [16], we expected to find the imidazolinone structure 3 (Fig. 3), which is similar to the methylglyoxal-arginine derivative 1 (Fig. 2) and to the reaction product 2 (Fig. 2), isolated after incubation of *N*-methylguanidine with 3-desoxypentosulose [17]. However,

Fig. 4 Assignment of spectroscopic data from heteronuclear multiple-bond correlation (HMBC) experiments to structure elements: insert ①–③ are details of two-dimensional HMBC plots



NMR-data of the protonated molecule pointed to the imidazolidine structure 4 (Fig. 3). Characteristic of the proposed structure 4 were the correlations of the protons at C-1′ with two carbonyl-like C-atoms at C-2 and C-5 in a ^{1}H, ^{13}C heteronuclear multiple-bond correlation (HMBC) experiment (insert ① Fig. 4). In compound 3 (Fig. 3), the distance between the first protons at C-δ and the second carbonyl-like C-atom (C-4) is five bonds, including two heteroatoms, thus no signal would have been found in the HMBC experiment. Additional affirmations of structure 4 are the two cross-peaks between the protons at C-4 and the carbonyl-like C-atoms C-2 and C-5 (insert ② Fig. 4). The connection of the hydrocarbon chain to the ring is confirmed by the cross-peak between the diastereotopic H-atoms at C-2′ and N-1 in a ^{1}H, ^{15}N HMBC experiment (insert ③ Fig. 4). Assignment of the N-atoms and their corresponding protons was carried out with a ^{1}H, ^{15}N heteronuclear multiple quantum coherence (HMQC) experiment. Here peak integration revealed that the exocyclic N-atom at C-2 carries *two* protons and the N-3 *one* proton, and these protons give $^{15}N/^{1}H$-HBMC signals with N-1 (data not shown). The chemical shifts of the N-atoms (Table 1) showed that the double bond within the ring system is located between N-1 and C-2. Thus, for the deprotonated form of the condensation product the 2-iminohydantoine structure 4 (Fig. 3), 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine, could be proposed.

Studies on the formation of "Glarg" in model mixtures showed that it is formed quickly under physiological conditions. An excess of glyoxal led to the complete derivatization of arginine, and even when arginine was incubated with glyoxal in the molar ratio of 1:1 a loss of at least 90% of the arginine peak area could be observed (t = 1 h, T = 37 °C, pH = 7.4). However, at higher temperatures (> 80 °C) the amount of "Glarg" decreases, and until now its reaction products have not been identified. Since it is not stable under conventional acid hydrolysis (23 h, 110 °C, 6N HCl), the detection of protein-bound "Glarg" had to be done after enzymatic hydrolysis of the samples.

To verify the formation of "Glarg" from protein-bound arginine, we incubated β-casein with varying quantities of glyoxal and noticed a direct correlation between the amount of added glyoxal and the "Glarg" peak area. In addition, the initially detectable arginine content in the β-casein decreased when more glyoxal was added (Fig. 1b). We also noticed a loss of lysine and the formation of carboxymethyllysine, as has already been shown [13]. More detailed studies on the protein–glyoxal interaction will be presented in a subsequent paper.

Compound 4 (Fig. 3), 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine, represents a new post-translational protein modification. Glyoxal is found in a great variety of foods [4], especially in those that are fermented. Therefore, the formation of protein-bound "Glarg" during food processing or storage should be expected. Furthermore, as α-dicarbonyls such as glyoxal are also thought to be produced by degradation of carbohydrates under physiological conditions, it seem likely that "Glarg" is present in biological systems.

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Reaction of 3-deoxypentosulose with *N*-methyl- and *N,N*-dimethylguanidine as model reagents for protein-bound arginine and for creatine

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Abstract. Deoxyosones are established key-intermediates in Maillard processes. Due to their dicarbonyl structure, they undergo condensation to form heterocyclic compounds with guanidine derivatives. In biological systems, guanidino functions are present in protein-bound arginine moieties as well as in creatine. The reactivity of such structures towards 3-deoxypentosulose is investigated with *N*-methyl- and *N,N*-dimethylguanidine as model substrates. Two diastereoisomers each are isolated from both reactions; they have been characterized unequivocally, respectively, as 4-(2,3-dihydroxypropyl)-2-*N*-methylamino-2-imidazolin-5-one and 4-hydroxy-5-(2,3-dihydroxypropyl)-2-(*N,N*-dimethylamino)-5*H*-imidazole. In aqueous medium as well as in the crystalline state, both diastereoisomer pairs exist in different tautomeric forms.

Introduction

A number of potential carbohydrate substrates are present in foodstuffs for the reaction with amino acids or protein-bound amino acid moieties, which is known as the Maillard reaction or "nonenzymatic browning". Modification of amino acids is frequently observed when proteins are heated in the presence of reducing carbohydrates; especially lysine and arginine undergo extensive derivatization in the course of nonenzymatic browning [1, 2]. Guanidino functions, however, have been reported not to initiate Maillard reactions [3]. If glucose, for instance, is heated together with N^{α}-acetylarginine, no browning of the solution is observed, i.e. the sugar molecule remains more or less unchanged [1, 2]. The guanidino function of an arginine moiety, incorporated in a protein, may be supposed, therefore, to react only with the more reactive Maillard intermediates, such as deoxyosones.

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The deoxyosones (compounds 1–3), formed in the course of the Maillard process by conversion of reducing pentoses and hexoses, respectively, may be characterized as intramolecular disproportionation products (see Fig. 1). They are much more reactive than the native carbohydrates.

From the reaction of a guanidino group with the α-dicarbonyl function of the 3-deoxyosones (compounds 1a, b), formation of imidazolinones may be envisaged, for which various tautomeric forms can be formulated (compounds 4–8; Fig. 2).

Likewise, an equilibrium is possible between the α- and β-dicarbonyl tautomeric form of the 1-deoxydiketoses (compounds 3a, b; Fig. 1), and thence formation of pyrimidine derivatives (compounds 9a, b; Fig. 3) [4].

Maillard reactions in food, as well as in vivo, are investigated predominantly with model substrates where undesirable reaction pathways are blocked, and isolation, purification and identification procedures are much simplified. The results thus obtained can be extrapolated to reactions in food and in the human organism.

In biological systems, guanidino groups are present in both the amino acid arginine (compound 10) and in the characteristic meat constituent creatine (compound 11; Fig. 4). We now report on products formed from the reaction of 3-deoxypentosulose (compound 1a) with *N*-methyl- and *N,N*-dimethylguanidine (compounds 12 and 13, respectively) as model substrates for protein-bound arginine and for creatine (Fig. 4).

Materials and methods

General methods

Spectra. IR spectra (KBr discs) were measured with Pye Unicam SP 1100 (Cambridge, UK) and Perkin Elmer 283 (Überlingen, Germany) spectrometers, UV spectra with a Perkin Elmer Lambda 2; [^{1}H]NMR and [^{13}C]NMR spectra were recorded on a Bruker (Karlsruhe, Germany) AC-250 spectrometer at 250 MHz and 63 MHz nominal frequency, respectively; for liquid-secondary-ion mass spectrometry (analogous to FAB-MS) a Finnigan MAT 95 (Bremen, Germany) was employed.

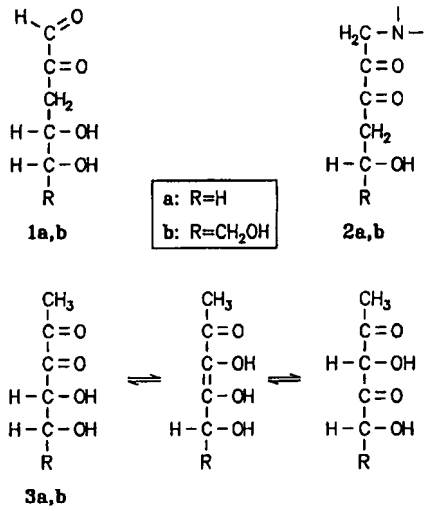


Fig. 1. Chemical structure of compounds 1–3: 3-deoxypentosulose (compound 1a); 3-deoxyhexosulose (compound 1b); *N*-substituted-1-amino-1,4-dideoxypentodiulose (compound 2a); *N*-substituted-1-amino-1,4-dideoxyhexodiulose (compcund 2b); 1-deoxypentodiulose (α-diketo, reductone, β-diketo structure) (compound 3a); 1-deoxyhexodiulose (α-diketo, reductone, β-diketo structure) (compound 3b)

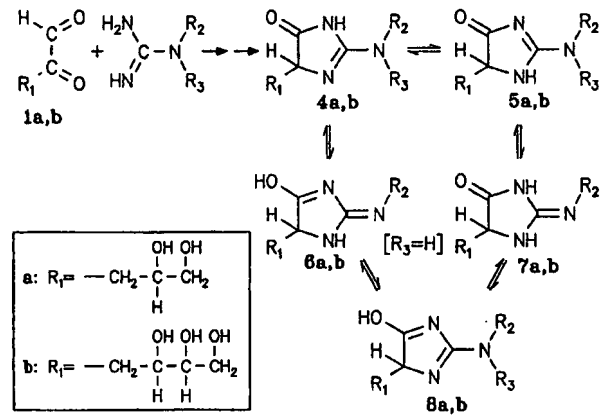


Fig. 2. Tautomeric forms (compounds 4–8) for the imidazolinones, formed by reaction of compound 1a, b with guanidine derivatives: 2-(di)alkylamino-4-(2,3-dihydroxypropyl)-2-imidazolin-5-one (compound 4a); 2-(di)alkylamino-4-(2,3,4-trihydroxybutyl)-2-imidazolin-5-one (compound 4b); 2-(di)alkylamino-5-(2,3-dihydroxypropyl)-2-imidazolin-4-one (compound 5a); 2-(di)alkylamino-5-(2,3,4-trihydroxybutyl)-2-imidazolin-4-one (compound 5b); 2-alkylimino-4-hydroxy-5-(2,3-dihydroxypropyl)-3-imidazoline (compound 6a); 2-alkylimino-4-hydoxy-5-(2,3,4-trihydroxybutyl)-3-imidazoline (compound 6b); 2-alkylimino-5-(2,3-dihydroxypropyl)imidazolidin-4-one (compound 7a); 2-alkylimino-5-(2,3,4-trihydroxybutyl)imidazolidin-4-one (compound 7b); 2-(di)alkylamino-4-hydroxy-5-(2,3-dihydroxypropyl)-5*H*-imidazole (compound 8a); 2-(di)alkylamino-4-hydroxy-5-(2,3,4-trihydroxybutyl)-5*H*-imidazole (compound 8b)

Semipreparative HPLC purification. A Merck Hitachi 655A-11 liquid chromatograph (Merck, Darmstadt, Germany) was used, combined with a 655A variable wavelength detector and a Bischoff (Leonberg, Germany) HPLC column (SNC + SPC Lichrosorb RP 18 – 5 μm; 8 × 250 mm): flow rate 2 ml·min^{-1}, detection at 220 nm.

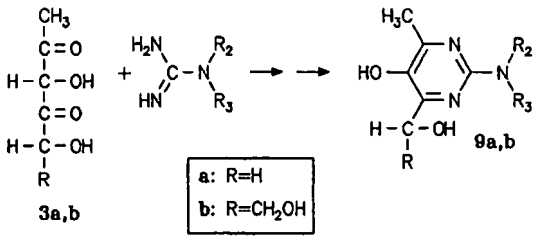


Fig. 3. Postulated formation of 2-(di)alkylamino-5-hydroxy-4-(2,3-dihydroxypropyl)-6-methylpyrimidine (compound 9a) and 2-(di)alkylamino-5-hydroxy-4-(2,3,4-trihydroxybutyl)-6-methylpyrimidine (compound 9b), respectively, from compound 3a, b and guanidine derivatives

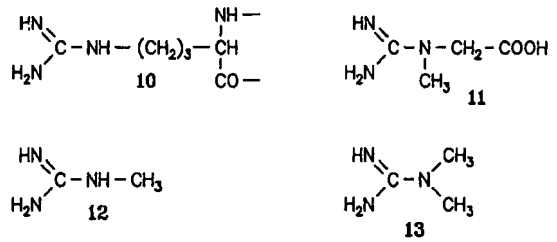


Fig. 4. Chemical structures of: protein-bound arginine (compound 10); creatine (compound 11); *N*-methylguanidine (compound 12); *N*,*N*-dimethylguanidine (compound 13)

Preparative HPLC purification. A Knauer (Berlin, Germany) 64 liquid chromatograph was employed, combined with an A0293 variable wavelength detector and a Kronlab (Sinsheim, Germany) HPLC column (guard column 20 × 50 mm, column 20 × 250 mm; Nucleosil RP 18 – 7 μm): flow rate 10 ml·min^{-1}, detection at 220 nm.

Capillary gas chromatograms. These were run on a Perkin-Elmer 8600 instrument with a flame-ionization detector (FID); quartz capillary column (length 25 m, ID = 0.32 mm, PVMS 54, film thickness = 1 μm, carrier gas = He, initial pressure = 80 kPa, 33 cm·s^{-1}); the temperature of the injection and detection ports was 270 °C; temperature programme was such that temperature rose at 8 °C·min^{-1} from 100 °C to 270 °C at which temperature it was held for 15 min.

Gas chromatography – mass spectrometry analysis. GC/MS was performed on a Finnigan MAT Ion Trap 800, EI and pos. CH_3OH–CI mode, coupled to a Perkin-Elmer 8420 gas chromatograph: quartz capillary column (length = 25 m, ID = 0.25 mm, PVMS 54, film thickness = 0.3 μm, carrier gas = He, initial pressure = 80 kPa, 21 cm·sec^{-1}); injection port temperature was 270 °C; the temperature programme was as mentioned above.

Chromatography. Silica gel 60 F_{254} Merck, 5554 and 5717 (Darmstadt, Germany) was used for thin-layer chromatography (TLC).

Lyophilization. A Leybold-Heraeus (Cologne, Germany) Lyovac GT 2 was applied.

Trimethylsilyl derivatives. These were obtained by treating 1 mg of the respective substrate with 100 μl *N*,*O*-bis(trimethylsilyl)acetamide (BSA) in 100 μl pyridine for 60 min at room temperature.

Materials

N-Methylguanidine·HCl (no. 22,240-2) and *N*,*N*-dimethylguanidine-sulphate (no. 27,666-9) were obtained from Aldrich

(Milwaukee, Wis., USA); *N,O*-bis(trimethylsilyl)acetamide (no. 15241) from Fluka (Neu-Ulm, Germany).

Synthetic procedures

Synthesis of 4-(2,3-dihydroxypropyl)-2-N-methylamino-2-imidazolin-5-ones (compounds 17a, b). A total of 250 mg (1.89 mmol) of 3-deoxypentosulose (compound 1a), synthesized according to [5], and 207 mg (1.89 mmol) *N*-methylguanidine·HCl were dissolved in 5 ml water, and the pH adjusted to 7.0 with 0.01 N NaOH. The solution was heated under reflux conditions for 4.5 h and the disappearance of compound 1a in the course of the reaction was monitored by TLC (eluent: ethylacetate:methanol 19:1; detection with 2,4-dinitrophenylhydrazine). After filtration (membrane filter 0.45 µm), the two diastereoisomers, compounds 17a and b, were separated by preparative HPLC (eluent: 0.01 M NH_4HCOO-buffer, pH = 5.0: CH_3CN 99.5:0.5; 6 injections, 1 ml each). The fraction with t_R (prep. HPLC) 8.5 and t_R 10.3 yielded, after lyophilization, 45 mg (0.24 mmol; 12.7%) of compound 17a and 35 mg (0.19 mmol; 9.9%) of compound 17b, respectively; GC t_R values of the silyl derivatives were 21.9 for compound 17a and 20.8 for compound 17b. According to the capillary gas chromatograms, fraction t_R 8.5 contains both diastereoisomers in a 3:1 ratio (17a:17b). Fraction t_R 10.3 contains only the diastereoisomer compound 17b.

UV absorbance, IR characteristics, and FAB-MS data of compounds 17a and b are identical:
1. UV (H_2O): maximum absorption wavelength (λ max) lg molar absorption coefficient (lg ε) = 192 nm (3.98); sh ≈ 215.
2. IR (KBr disc): peaks were measured at: 3200 (broad), 1710, 1615, 1410, 1355, 1115, 1070 cm^{-1}
3. FAB-MS (*m*-nitrobenzyl alcohol): 188 (M + H).

For compound 17a, the following characteristics apply:
1. CI-GC/MS [tetrakis(trimethylsilyl) derivative]: m/z 476(M + H; 100) 460(26) 404(4) 257(26) 73(14).
2. [^{1}H]NMR (^2H_2O): (chemical shifts (δ, ppm) 2.01 [ddd, 1H, J = 4.5, 10.8, (−)14.8 Hz] δ 2.17 [ddd, 1H, J = 3.2, 6.0, (−)14.8 Hz] δ 3.18 (s, 3H) δ 3.49 [dd, 1H, J = 6.3, (−)11.8 Hz] δ 3.57 [dd, 1H, J = 4.2, (−)11.8 Hz] δ 3.90 (dddd, 1H, J = 3.2, 4.2, 6.3, 10.8 Hz) δ 4.55 (dd, 1H, J = 4.5, 6.0 Hz), where J is the coupling constant (Hz), S = singlet, d = doublet, dd = double doublet, ddd = double double doublet, t = triplet.
3. [^{13}C]NMR (^2H_2O): δ 28.6, 35.7, 59.2, 68.1, 70.9, 161.6, 178.6.

For compound 17b the following characteristics apply:
1. CI-GC/MS [tris(trimethylsilyl) derivative]: m/z 404(M + H; 100) 388(12) 198(7) 73(49).
2. [^{1}H]NMR (^2H_2O): δ 1.89 [ddd, 1H, J = 3.6, 9.8, (−)14.6 Hz] δ 2.03 [ddd, 1H, J = 3.7, 9.7, (−)14.6 Hz] δ 3.19 (s, 3H) δ 3.54 [dd, 1H, J = 6.2, (−)11.7 Hz] δ 3.60 [dd, 1H, J = 4.4, (−)11.7 Hz] δ 3.86 (dddd, 1H, J = 3.6, 4.4, 6.2, 9.7 Hz] δ 4.58 (dd, 1H, J = 3.7, 9.8 Hz).
3. [^{13}C]NMR (^2H_2O): δ 28.6, 36.7, 58.8, 68.1, 70.8, 161.7, 178.4.

Synthesis of 4-Hydroxy-5-(2,3-dihydroxypropyl)-2-(N,N-dimethylamino)-5H-imidazoles (compounds 18a, b). Following the above described procedure, 250 mg (1.89 mmol) 3-deoxypentosulose (compound 1a) and 350 mg (1.89 mmol) *N,N*-dimethylguanidine·H_2SO_4 were heated under reflux conditions for 3 h. The membrane filtrate was separated by semipreparative HPLC (eluent: 0.01 M NH_4HCOO-buffer pH = 5.0:CH_3CN 99:1; 25 injections, 50 µl each). The fraction with t_R (semiprep. HPLC) 14.3 and t_R 18.8 yielded, after lyophilization, 14 mg crude compound 18a and 7 mg of compound 18b, respectively. The purity of both fractions could not be analysed by GLC; the peak shapes indicated decomposition of the silyl derivatives. Further analysis by semipreparative HPLC showed that fraction t_R 18.8 contained pure compound 18b. Fraction t_R 14.3, in contrast, contains both diastereoisomers in a 2:1 ratio (18a:18b). The lyophilized fraction t_R 14.3 was dissolved in 1 ml water and once again subjected to semipreparative HPLC to obtain pure compound 18a (eluent as mentioned above; 10 injections, 100 µl each). Fractions t_R 14.3 and 18.8 were collected and lyophilized, yielding 8.5 mg (0.042 mmol; 8.9%) compound 18a and 10 mg (0.05 mmol; 10.6%) compound 18b.

UV absorbance, IR characteristics, and FAB-MS data of compounds 18a and b are identical:
1. UV (H_2O): λ max(lg ε) = 225 nm (4.15).
2. IR (KBr disc) peaks were measured at: 3360, 3110, 1615, 1440, 1390, 1318, 1290, 1080 cm^{-1}
3. FAB-MS (*m*-nitrobenzyl alcohol): 202 (M + H).

For compound 18a, the following characteristics apply:
1. [^{1}H]NMR (^2H_2O): δ 1.84 [ddd, 1H, J = 6.0, 9.5, (−)14.7 Hz] δ 2.06 [ddd, 1H, J = 4.0, 5.5, (−)14.7 Hz] δ 3.09 (s, 3H) 3.16 (s, 3H) δ 3.52 [dd, 1H, J = 6.5, (−)11.8 Hz] δ 3.61 [dd, 1H, J = 4.1, (−)11.8 Hz] δ 3.92 (ddt, 1H, J = 4.0, 6.5, 9.5 Hz) δ 4.32 (dd, 1H, J = 5.5, 6.0 Hz).
2. [^{13}C]NMR (^2H_2O): δ 37.1, 39.4, 41.1, 62.2, 68.3, 71.8, 170.2, 193.3.

For compound 18b, the following characteristics apply:
1. [^{1}H]NMR (^2H_2O): δ 1.72 [ddd, 1H, J = 3.2, 9.4, (−)14.5 Hz] δ 1.93 [ddd, 1H, J = 3.7, 9.8, (−)14.5 Hz] δ 3.07 (s, 3H) 3.15 (s, 3H) δ 3.53 [dd, 1H, J = 6.5, (−)11.7 Hz] δ 3.60 [dd, 1H, J = 4.2, (−)11.7 Hz] δ 3.84 (dddd, 1H, J = 3.2, 4.2, 6.5, 9.8 Hz) δ 4.34 (dd, 1H, J = 3.7, 9.4 Hz).
2. [^{13}C]NMR (^2H_2O): δ 38.1, 39.4, 41.1, 62.1, 68.5, 71.7, 172.0, 195.4.

Results and discussion

For 3-deoxypentosulose (compound 1a), an equilibrium may be envisaged between the various cyclic isomers (compounds 14–16; Fig. 5), with the open-chain form, compound 1a, as only a minor constituent. Since for the intramolecular hemiacetal/ketal structures 14–16 much lower α-dicarbonyl activity is expected, cyclization reactions with guanidine derivatives may be retarded significantly relative to those of proper α-dicarbonyl compounds, such as benzil or methylglyoxal.

We heated equimolar aqueous solutions of compound 1a and *N*-methylguanidine·HCl (compound 12) or *N,N*-dimethylguanidine·H_2SO_4 (compound 13) under reflux conditions for 3–4.5 h to simulate cooking conditions. The pH, adjusted to 7.0 at the outset of the reaction, decreases to 3.5–4 during the heating period, and the reaction mixtures turn dark brown. The pH decrease may be due to carboxylic acids being formed by degradation of

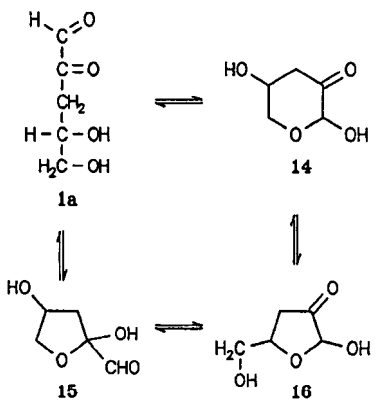


Fig. 5. Hemiacetals/ketals of compound 1a: 2,5-dihydroxytetrahydro-3-pyranone (compound 14); 2,4-dihydroxytetrahydrofuran-2-carbaldehyde (compound 15); 2-hydroxy-5-hydroxymethyl-4,5-dihydro-3(2*H*)-furanone (compound 16)

compound 1a. However, the decidedly lower basicity of the imidazolinone products, relative to that of the starting guanidine derivatives, may play an even more important role.

After membrane filtration, the solutions are subjected directly to either preparative or semipreparative HPLC. Two diastereoisomers each are isolated from both the reactions with compounds 12 and 13. Spectroscopic data (see below) unequivocally prove the formation of the diastereoisomeric 4-(2,3-dihydroxypropyl)-2-*N*-methylamino-2-imidazolin-5-ones (compounds 17a, b) from the reaction with *N*-methylguanidine (compound 12), and of the diastereoisomeric 4-hydroxy-5-(2,3-dihydroxypropyl)-2-(*N*,*N*-dimethylamino)-5*H*-imidazoles (compounds 18a, b) from reaction with *N*,*N*-dimethylguanidine (compound 13). A plausible reaction mechanism for formation of compounds 17a, b and compounds 18a, b is outlined in Fig. 6. An intramolecular Cannizzaro reaction may safely be excluded under Maillard conditions, i.e. pH 4–7. The proton at C4 in compound 17a, b, and at C5 in compound 18a, b, therefore must be solvent-derived, and does not stem from a 1,2-hydride shift.

The yield of compounds 17a, b and 18a, b from the reaction of 3-deoxypentosulose (compound 1a) with the respective guanidine derivative is about 20% for each diastereoisomer pair. Formation of these compounds thus constitutes a major reaction pathway for 3-deoxypentosulose (compound 1a) derivatization in the presence of guanidino moieties. Investigations are now in progress to study formation of compunds 17a, b and 18a,.b under physiological conditions. Oxidation of such structures would give very reactive imidazolinone systems.

There are several reports in the literature about the reaction of α-dicarbonyl compounds with guanidine derivatives. Nishimura and Kitajima [6] describe 4-hydroxy-2-dimethylamino-4,5-diphenyl-4*H*-imidazole (compound 19) being formed from benzil and *N*,*N*-dimethylguanidine (compound 13) at room temperature (Fig. 7). If heated in either *N*,*N*-dimethylformamide (DMF), ethanol, or in neat form, compound 19 is transformed quantitatively into an imidazolinone (compounds 20 or 21). Mainly on the basis of the C=O stretching frequency, the authors favour the tautomeric form, compound 20, in the solid state.

Duerksen-Hughes et al. [7] have studied the reaction of guanidine derivatives with 4-(oxoacetyl)phenoxyacetic acid. Reaction (37 °C, basic conditions) of *N*-methylguanidine (compound 12) with 4-(oxoacetyl)phenoxyacetic acid yields 4-(2-methylimino-4-imidazolidinon-5-yl)phenoxyacetic acid (compound 22; Fig. 7). Structure 22, with an exocyclic imino group, was assigned from [^{1}H]NMR and [^{13}C]NMR spectra interpretation.

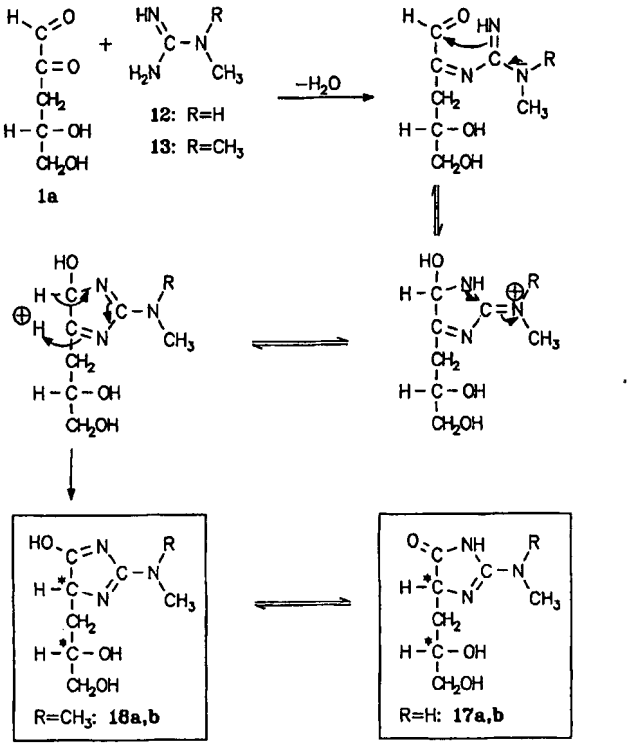


Fig. 6. Hypothetical reaction mechanism for the formation of the two diastereoisomeric 4-(2,3-dihydroxypropyl)-2-*N*-methylamino-2-imidazolin-5-ones (compounds 17a, b) and 4-hydroxy-5-(2,3-dihydroxypropyl)-2-(*N*,*N*-dimethylamino)-5*H*-imidazoles (compounds 18a, b)

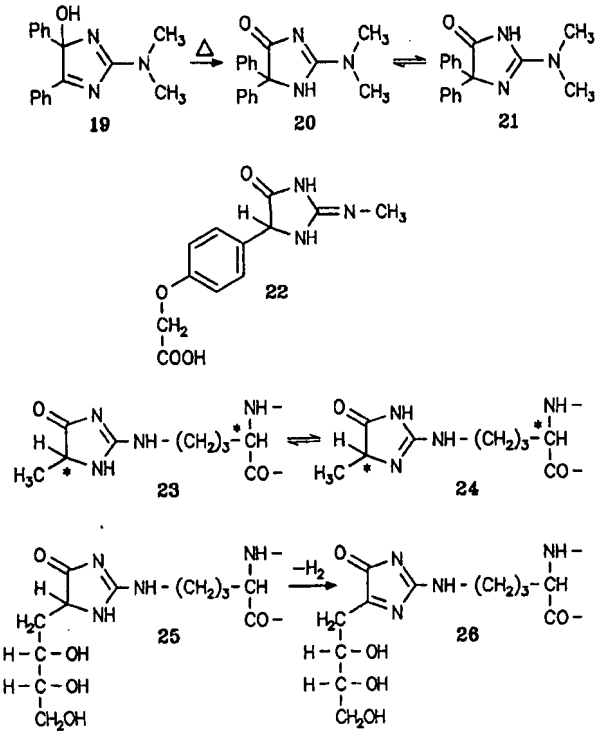


Fig. 7. Diazole structures from reactions of α-dicarbonyl compounds and guanidine derivatives as described in the literature: 2-*N*,*N*-dimethylamino-4-hydroxy-4,5-diphenyl-4*H*-imidazole (compound 19); 2-*N*,*N*-dimethylamino-5,5-diphenyl-2-imidazolin-4-one (compound 20), 2-*N*,*N*-dimethylamino-4,4-diphenyl-2-imidazolin-5-one (compound 21), 4-(2-methylimino-4-imidazolidinon-5-yl)phenoxyacetic acid (compound 22), 5-methyl-2-(δ-*N*-ornithyl)-2-imidazolin-4-one (compound 23), 4-methyl-2-(δ-*N*-ornithyl)-2-imidazolin-5-one (compound 24), 5-(2,3,4-trihydroxybutyl)-2-(δ-*N*-ornithyl)-2-imidazolin-4-one (compound 25)5-(2,3,4-trihydroxybutyl)-2-(δ-*N*-ornithyl)-4-imidazolone (compound 26)

Thornalley [8] has postulated that 5-methyl-2-(δ-*N*-ornithyl)-2-imidazolin-4-one (compound 23) is a fluorescent reaction product formed from methylglyoxal and protein-bound arginine (compound 10; Fig. 7). Henle et al. [9] have isolated compound 23 and the tautomeric 4-methyl-2-(δ-*N*-ornithyl)-2-imidazolin-5-one (compound 24) from a food sample, and subsequently synthesized this structure independently. One diastereoisomer only was detected for each tautomer from both the foodstuff and the independent chemical reaction. Structural assignment of compounds 23 and 24 is based on the [^{1}H]NMR, [^{13}C]NMR, and FAB-MS data.

Hayase et al. [10, 11] reported on the formation of 5-(2,3,4-trihydroxybutyl)-2-(δ-*N*-ornithyl)-2-imidazolin-4-one (compound 25) and its oxidation product 5-(2,3,4-trihydroxybutyl)-2-(δ-*N*-ornithyl)-4-imidazolone (compound 26) from 3-deoxyhexosulose (compound 1b) and protein-bound arginine (compound 10; Fig. 7); both products were characterized, after acid hydrolysis of the protein and chromatographic purification, by [^{1}H]NMR, [^{13}C]NMR, and FAB-MS.

Formation of 2-alkylamino-imidazolinone-type structures from α-dicarbonyl compounds and guanidine derivatives thus may be considered to be firmly established. Which tautomeric form is present in each case, though, is discussed controversially in the literature references cited above – despite the fact that the individual structures will differ significantly in their UV, IR, and NMR characteristics. Therefore, we have put special efforts into an unequivocal structural assignment for our compounds 17a, b and 18a, b.

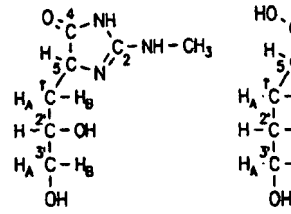
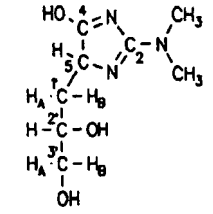
Structural assignment

The differentiation between the two tautomeric forms of the *N*-methyl- and *N,N*-dimethyl derivatives, compounds 17a, b and 18a, b respectively, rests on a number of individual spectroscopic arguments. Each of these, if taken alone, provides only circumstantial evidence; taken together, however, the two structures may be considered as firmly established.

[^{1}H] and [^{13}C] chemical shifts (δ) and coupling constants (J) or multiplicities are given in Table 1. The chemical shifts for carbons C4 and C2 differ strikingly between compounds 17a, b and 18a, b. In compounds 17a, b, the C4 shift (178.6/178.4 ppm) is in the range expected for a cyclic amide. This is confirmed by a 1710 cm^{-1} C=O stretching vibration, characteristic for a γ-lactam. The C2 resonances in compounds 17a, b (161.6/161.7 ppm) appear only slightly shifted to lower field compared to that of the starting alkyl guanidine (158 ppm); C2, therefore, cannot be conjugated with the carbonyl function. The UV absorption maximum (192 nm; ε ≈ 9500) likewise excludes extended conjugation, being characteristic, rather, for the π → π* transition of a structurally isolated imine group. The CH_3 carbon shift (28.6 ppm) for compounds 17a, b clearly proves the imine to be endocyclic; for an imine-bound CH_3 carbon, a chemical shift of about 39 ppm would be expected.

The C4 and C2 resonances for compounds 18a, b (193.3/195.4 ppm and 170.2/172.0 ppm, respectively) are shifted by 10–15 ppm to low field relative to those of compounds 17a, b. Such shifts are reasonable for a C=N conjugated enaminole structure. This hypothesis is supported by the IR spectrum which shows no proper C=O stretching vibration. The intensive band at 1615 cm^{-1} can be assigned to the coupled C=N stretching modes for the cyclic conjugated C=N functions. Such a diene structure for compounds 18a, b is also confirmed by the UV absorption maximum (225 nm; ε ≈ 14000).

Table 1. [^{1}H]NMR and [^{13}C]NMR spectroscopic data of compounds 17a, b and 18a, b in ^2H_2O (for a direct comparison between the two diazole structures, the same numbering is used for compounds 17a, b and 18a, b)

Compound structures				
				
17a	**17b**	**18a**	**18b**	
[^{1}H]NMR	δ (ppm)			
5-H	4.55	4.58	4.32	4.34
1'-H_A	2.01	1.89	1.84	1.72
1'-H_B	2.17	2.03	2.06	1.93
2'-H	3.90	3.86	3.92	3.84
3'-H_A	3.49	3.54	3.52	3.53
3'-H_B	3.57	3.60	3.61	3.60
CH_3	3.18	3.19	3.16	3.15
			3.09	3.07
	J (Hz)			
^2J(1'-H_A; 1'-H_B)	(−) 14.8	(−) 14.6	(−) 14.7	(−) 14.5
^2J(3'-H_A; 3'-H_B)	(−) 11.8	(−) 11.7	(−) 11.8	(−) 11.7
^3J(5-H; 1'-H_A)	4.5	9.8	6.0	9.4
^3J(5-H; 1'-H_B)	6.0	3.7	5.5	3.7
^3J(1'-H_A; 2'-H)	10.8	3.6	9.5	3.2
^3J(1'-H_B; 2'-H)	3.2	9.7	4.0	9.8
^3J(2'-H; 3'-H_A)	6.3	6.2	6.5	6.5
^3J(2'-H; 3'-H_B)	4.2	4.4	4.1	4.2
[^{13}C]NMR	δ (ppm)			
2C	161.6 (4)	161.7 (4)	170.2 (4)	172.0 (4)
4C	178.6 (4)	178.4 (4)	193.3 (4)	195.4 (4)
5C	59.2 (3)	58.8 (3)	62.2 (3)	62.1 (3)
1'C	35.7 (2)	36.7 (2)	37.1 (2)	38.1 (2)
2'C	70.9 (3)	70.8 (3)	71.8 (3)	71.7 (3)
3'C	68.1 (2)	68.1 (2)	68.3 (2)	68.5 (2)
CH_3	28.6 (1)	28.6 (1)	39.4 (1)	39.4 (1)
			41.1 (1)	41.1 (1)

δ, Chemical shift for the indicated bond; J coupling constant between the indicated bonds; (1), (2), (3), (4) denotes a primary, secondary, tertiary, or quaternary carbon atom, respectively, as indicated by the polarization in the [^{13}C]-DEPT NMR spectrum

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